Germ Cell Modulation of Sertoli Cell Function

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Contents

List of Figures

List of Tables

Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree.

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Abstract

Spermatogenesis is the process whereby A type spermatogonia undergo a series of cell divisions, meiosis and remodelling events to become spermatozoa. This germ cell development is thought to be dependent on the supply of various factors by the Sertoli cell. The function of the Sertoli cell changes dramatically according to the stage of the spermatogenic cycle, and there are many Sertoli cell products which are produced and/or secreted in a cyclical pattern (Parvinen, 1993). There is growing evidence that this cyclicity of function of the Sertoli cell is a consequence of the changing germ cell complement with which the Sertoli cell is associated at the different stages (Sharpe, 1993). The aim of this work was to assess the influence of germ cells on the expression of selected Sertoli cell mRNAs. To this end, adult rats were treated with 650mg/kg methoxyacetic acid (MAA) to induce the specific depletion of >80% of pachytene and later spermatocytes from most tubules, and expression of selected Sertoli cell mRNAs was then assessed at various times after treatment when particular germ cell types were depleted selectively (see Bartlett *et al.* 1988; Allenby *et al.*, 1991). Studies on the expression of cyclic protein 2 (CP-2) mRNA supported the hypothesis that germ cells can influence the cyclic function of Sertoli cells. Expression of CP-2 mRNA was shown by Northern blot analysis to decrease significantly 21 days after MAA treatment. *In situ* hybridisation showed that CP-2 mRNA expression was decreased or absent from tubules at stages at which CP-2 mRNA is normally expressed (stages IV-VII) when elongate spermatids were depleted selectively from these tubules. This decrease was reflected in loss of CP-2 protein production. These observations lead us to hypothesise that elongate spermatids positively modulate CP-2 expression in the Sertoli cell, with this modulation occuring at the level of transcription.

The level of expression of α-inhibin appeared to be negatively regulated by pachytenes spermatocytes and elongate spermatids, although these findings remain to be confirmed by *in situ* hybridisation. Germ cell regulation of Sertoli cell transferrin gene expression was investigated but results were complicated by the existence of a germ cell mRNA, hemiferrin. This transcript has extensive homology to transferrin and was detected by the initial cDNA probes used. No significant effect of germ cells on transferrin mRNA levels could be detected but this requires further investigation. The presence of cystatin C mRNA in germ cells was demonstrated as was tentative localisation to Sertoli cells. *In situ* hybridisation detected changes in the level of mRNA at all time points after MAA treatment due to germ cell depletion but was unable to discriminate between direct effects of germ cell depletion and an influence on Sertoli cell expression. Immunohistochemistry detected cystatin C predominantly in Sertoli cells suggesting that the mRNA expressed in the germ cells was not translated. Preliminary investigations using Northern blot analysis and *in situ* hybridisation failed to detect any effect of germ cell depletion on the expression of cellular retinol binding protein or androgen binding protein (ABP) mRNA in Sertoli cells. However, in the case of ABP an effect may occur post-transcriptionally as depletion of elongate spermatids has previously been shown to result in a decrease in ABP secretion (Pinon-Lataillade *et al.*, 1988).

Subtractive hybridisation was performed to isolate Sertoli cell genes which are regulated by elongate spermatids. Seventeen positive clones were isolated but preliminary studies failed to detect any change in their mRNA expression when elongate spermatids were depleted from seminiferous tubules. One clone was identified as having homology to part of the mitochondrial genome coding for tRNA-Met and NADH dehydrogenase subunit II. This is consistent with previous reports suggesting that genes encoded by mitochondrial DNA are frequently isolated during subtractive hybridisation procedures.

In conclusion, this study demonstrated that germ cells do appear to influence Sertoli cell gene expression. This can occur at the level of transcription as was demonstrated by the effect of elongate spermatids on CP-2 mRNA expression, or may occur post-transcriptionally, as would appear to be the case with ABP.

[approximately 52,000 words in main text]

Commonly Used Abbreviations

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CTP	Cytosine triphosphate
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
GTP	Guanosine triphosphate
hCG	Human chorionic gonadotropin
IPTG	Isopropyl β-D-thiogalactopyranoside
LH	Luteinising hormone
MOPS	3-[N-Morpholino]propane-sulphonic acid
PCR	Polymerase chain reaction
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rNTP	Ribonucleotide triphosphate
SDS	Sodium dodecyl sulphate
TEA	Triethanolamine
TEMED	N,N,N',N'-Tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane
TTP	Thymidine triphosphate
UTP	Uridine triphosphate
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galatoctopyraoside

1. Introduction

Our inability to treat the majority of cases of male infertility and the slow progress that has been made towards development of non-steroidal methods of male contraception is due in part to a basic lack of understanding of the cellular interactions involved in the process of sperm production. A recent review of all available literature on semen analysis of unselected healthy men has shown that total sperm counts have fallen by approximately 50% in the last 50 years (Carlsen *et al.*, 1992). In this time period the incidence of testicular cancer, which is associated with a decrease in semen quality, has risen (Osterlind, 1986). In addition, there are indications that there has been a parallel rise in other genitourinary abnormalities such as hypospadias and cryptorchidism (Giwercman & Skakkebaek, 1992). The underlying cause of this increase in disorders of development and function of the male reproductive tract is unknown but it has been suggested that exposure to chemicals in our environment may be having an adverse affect on spermatogenesis (Sharpe, 1992). However, another possibility gaining support is that these changes have been brought about by an increase in our exposure to oestrogens during foetal development (Sharpe & Skakkebaek, 1993). This could cause both disorders of the reproductive tract and a decrease in sperm count in adult life. Our current inability to define the underlying causes for this apparent decrease in sperm count again serves to highlight our ignorance of male reproductive physiology.

The aim of the work described in this thesis was to further our understanding of the complex events which make up the process of spermatogenesis. It is already well established that the normal production of sperm by the mammalian testis is dependent on an adequate supply of follicle-stimulating hormone (FSH) and luteinising hormone (LH) from the pituitary gland and on the LH-stimulated production of testosterone by Leydig cells. The regulation of the synthesis and secretion of FSH and LH, and the dependence of spermatogenesis on Leydig cell steroidogenesis are discussed in chapter 2. However, maintenance of quantitatively and qualitatively normal sperm production is also dependent on a series of interactions between the different cell types present in the testis (Sharpe *et al.*, 1993). It is known that Leydig cell steroidogenesis can be influenced by the production of proteins by the Sertoli cell (Sharpe *et al.*, 1981; Papadopoulos, 1991) and recent evidence has suggested that germ cells also have a role to play in indirectly regulating testosterone synthesis or secretion (Onoda *et al.*, 1991). It is well established that division of the immature germ cells and their differentiation to become mature spermatozoa is totally dependent on the provision of various factors by the Sertoli cells (Parvinen, 1993). In turn, the available evidence suggests that germ cells are involved in the regulation of Sertoli cell function (Sharpe, 1993).

The studies described in this thesis involved an investigation of germ cell modulation of Sertoli cell function. This was investigated using an *in vivo* model of germ cell depletion from adult rat testis by administration of a single oral dose of the testicular toxicant methoxyacetic acid. Chapter 4 describes preliminary investigations into the effect of germ cell depletion on the expression of specific mRNAs by Sertoli cells. These initial experiments led to a more detailed study of the possible germ cell regulation of transferrin and cyclic protein 2 (CP-2) mRNA expression by Sertoli cells as outlined in chapters 5 and 6, respectively. The final experimental chapter of this work describes the methodology used in an attempt to isolate novel genes expressed in Sertoli cells, the expression of which are positively modulated by the presence of elongate spermatids.

The aim of the studies described in this thesis is to increase our knowledge of the role germ cells have to play in indirectly controlling their own growth and differentiation by the modulation of Sertoli cell function. This data will add to the growing number of studies on the importance of germ cell-Sertoli cell interactions in the maintenance of normal testicular function. It is hoped that studies such as these will increase our understanding of the normal process of spermatogenesis and its control so that we can identify cellular events underlying some of the cases of male infertility and allow the design of more effective therapies or novel contraceptive strategies.

2. Review of the Literature

2.1. Introduction

The testis has two main functions; 1) the synthesis of the male sex hormone, testosterone, and 2) the daily production of millions of spermatozoa. These functions are necessary for determining both masculinity and fertility and are essential for the continuation of the species.

The endocrine function of the testis was first demonstrated by Professor Berthold of Göttingen in 1849. He showed that removal of the testis from cockerels resulted in under-development of their combs and a change in their behaviour; the birds were no longer aggressive and showed no interest in hens. However, if the testes were not removed but their nervous and vascular connections broken by transplantation to the abdomen the birds retained normal cockerel behaviour. These experiments led Berthold to conclude

"...the testes affect the blood and then by corresponding effects of the blood they can affect the entire organism...".

Leydig cells were first shown to be the site of production of this masculising factor in 1903 (Bouin & Ancel, 1903) but testosterone was not finally isolated from testicular tissue until 1935 (David *et al.*, 1935).

The importance of the pituitary gland in the regulation of testicular function was established in 1930 (Smith, 1930). This study showed that hypophysectomy of adult male rats caused regression of the seminiferous epithelium and a decrease in size of the reproductive organs. The rats also showed a complete loss of libido. However, spermatogenesis was restored and all other functions were reinitiated by the daily replacement of portions of pituitary gland to these rats. The importance of two pituitary hormones, luteinising hormone (LH) and follicle stimulating hormone (FSH), was shown in 1936 when Greep and co-workers observed that injection of FSH and LH into immature hypophysectomised male rats caused development of the seminiferous epithelium and the interstitial tissue, respectively(Greep *et al.*, 1936).

The importance of both endocrine and paracrine factors in the regulation of testicular function has been shown conclusively in numerous studies and the relative contribution of these systems is discussed in the following sections.

2.2. Organisation of the testis

The testis is organised into two main compartments, the seminiferous tubules and the interstitial tissue (see Fig.1). It is in these compartments that the two main functions of the testis occur, the synthesis of androgens by the Leydig cells of the interstitium and the production of spermatozoa by the seminiferous tubules. The cells involved in these processes are extremely specialised and are described in the following sections.

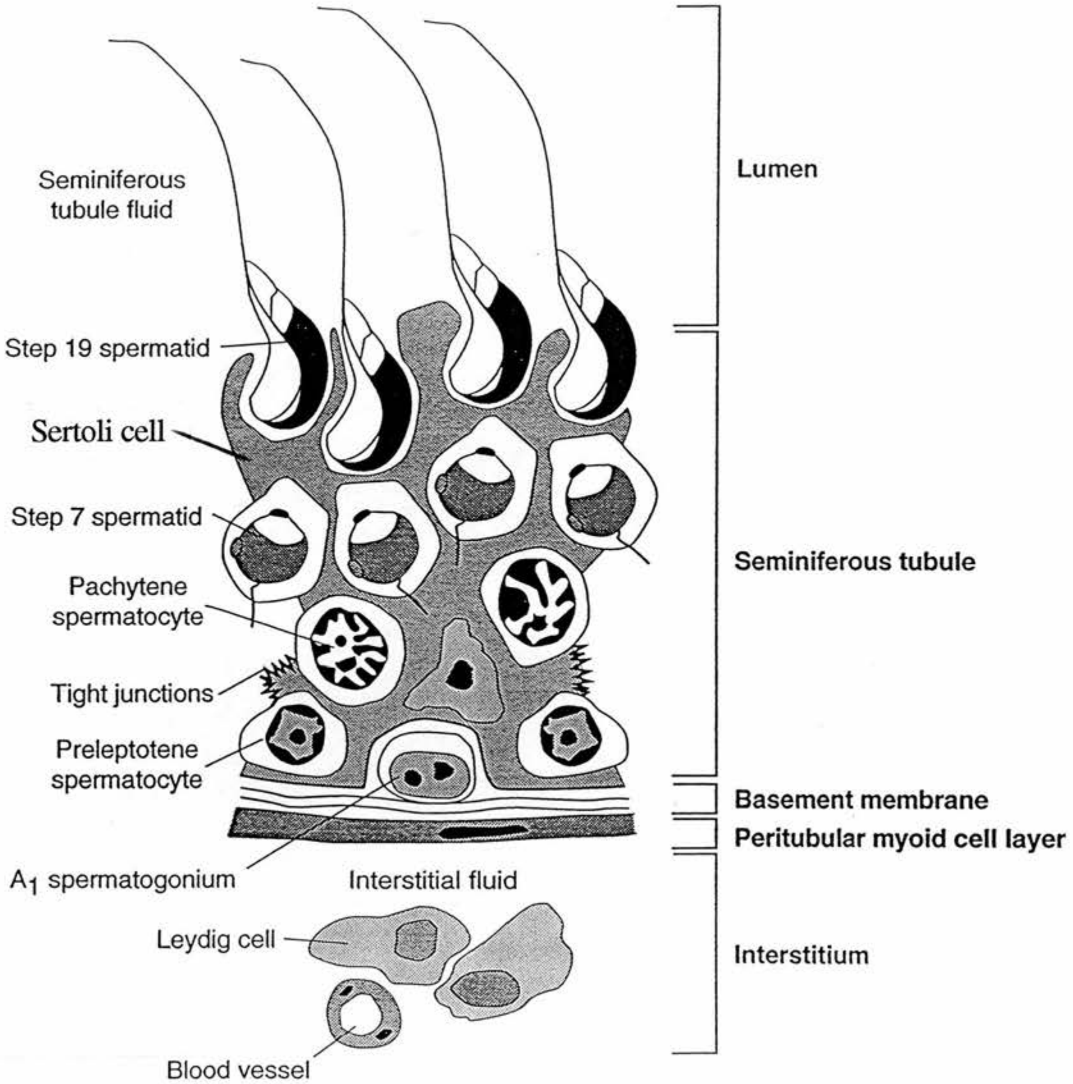


Figure 1. Compartmentalisation of the testis. Figure shows the cellular associations of a seminiferous tubule at stage VII of the spermatogenic cycle and the cells of the interstitial space with which it is in contact. *Adapted from Sharpe, 1994.*

2.2.1 The Seminiferous Epithelium

The seminiferous tubule is comprised of a seminiferous epithelium, basement membrane and peritubular cells. The cells within the seminiferous epithelium are arranged in a highly organised manner. In the rat, cross sections of seminiferous tubules show a fixed complement of 4 or 5 types of germ cells which are in intimate contact with the Sertoli cells. These associations have been divided into fourteen different stages which taken together make up the spermatogenic cycle (Fig.2; Leblond and Clermont, 1952). In man there are only six recognised stages of the spermatogenic cycle and in one length of tubule the stages are mixed because of a helical rather than a segmental arrangement of the stages along the length of the seminiferous epithelium (Schulze & Rehder, 1984).

2.2.1.1 Spermatogenesis

Spermatogenesis is the process whereby A type spermatogonia undergo a series of cell divisions, meiosis and remodelling events to become spermatozoa. Development of the germ cells takes place within the seminiferous epithelium. In the rat, each germ cell passes through the 14 stages of the spermatogenic cycle 4.5 times. The whole process from the division of the spermatogonial stem cells to the release of the mature spermatid from the seminiferous epithelium takes 56 days in the rat (Clermont & Harvey, 1965) and approximately 70 days in man (Schulze & Rehder, 1984).

Spermatogenesis can be considered as being composed of **three** main events namely, 1) **proliferation** of the spermatogonia, 2) **meiosis** to produce haploid round spermatids and 3) **spermiogenesis**, the process whereby round spermatids differentiate into morphologically mature spermatozoa. In the rat the stem cell, or A_o type spermatogonia undergo a series of mitotic divisions at specific times during the spermatogenic cycle to give rise to A_1, A_2, A_3, A_4 and finally B type spermatogonia (Clermont, 1962). In man the type A spermatogonia do not undergo as many replications as in the rat which may account in part for the less efficient spermatogenesis found in humans (Paniagua *et al.*, 1987). B type spermatogonia undergo a final mitotic division and then enter a lengthy meiotic division as primary spermatocytes involving DNA and RNA synthesis at specific times in their development and finally giving rise to four haploid spermatids for each original spermatocyte assuming no germ cell degeneration occurs (Monesi *et al.*, 1978). Haploid spermatids then enter the final phase of spermatogenesis which involves development of the acrosome, elongation of the spermatid and nuclear condensation (Parvinen, 1982). As the spermatid develops the acrosome becomes associated with the

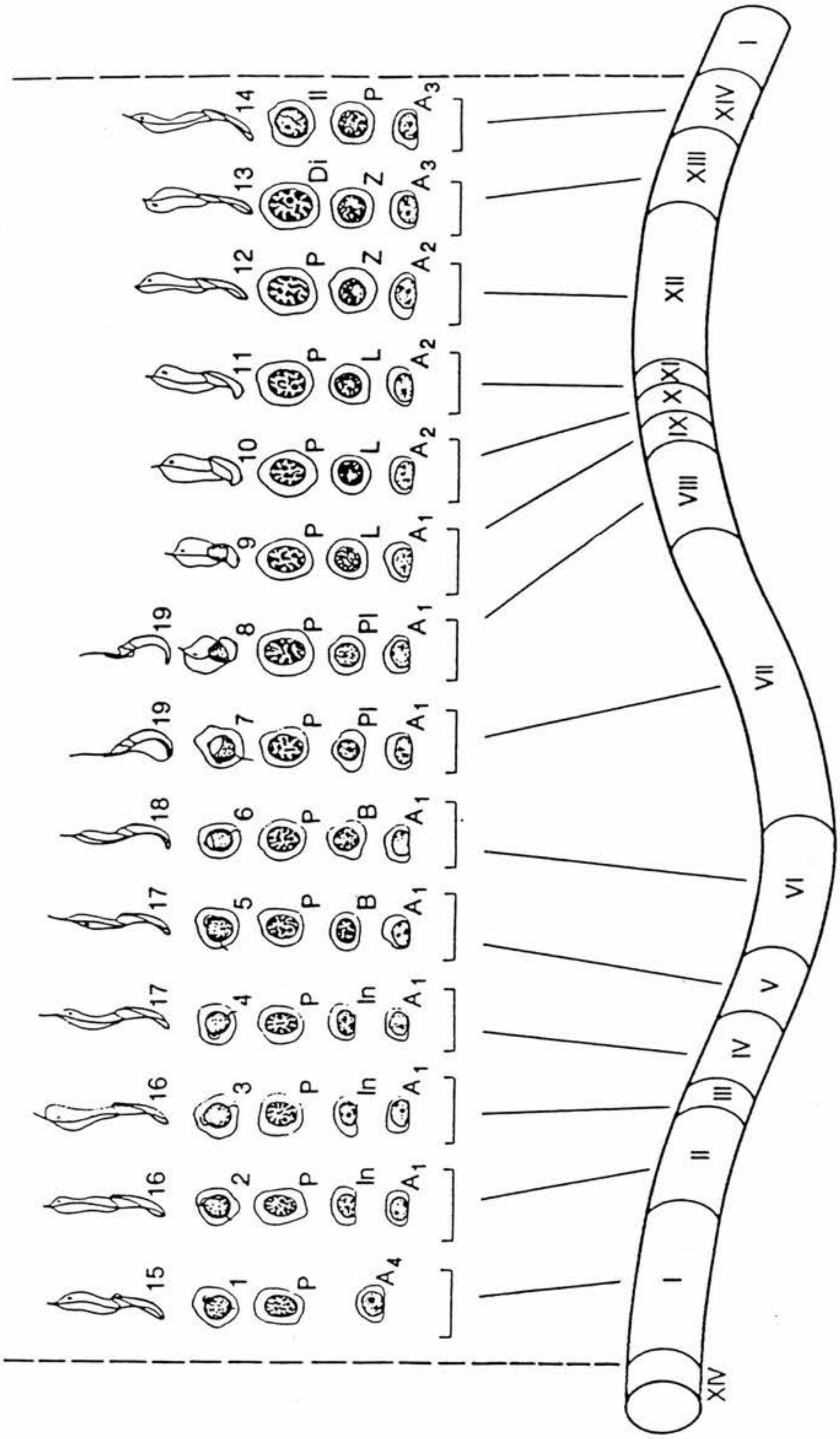


Figure 2. The spermatogenic cycle of the rat. The figure shows the fourteen stages of the spermatogenic cycle in a helical arrangement along the length of the seminiferous tubule. The germ cells associated with Sertoli cells at each stage of the cycle are shown. A_1-A_4, In and B = A, intermediate and B type spermatogonia; Pl, L, Z and P = preleptotene, leptotene, zygotene and pachytene spermatocytes; 1-19 = round and elongating spermatids. *Adapted from Parvinen, 1982.*

nuclear envelope and spreads over its surface. RNA synthesis ceases in step 8 spermatids (Monesi *et al.*, 1978), the nuclear chromatin begins to condense and elongation of the nucleus is observed. This involves changes in nuclear DNA binding proteins, with the histones found in early spermatids being replaced first by transition proteins (Heideran & Kistler, 1987; Kleene & Flynn, 1987) and then by protamines which are present in the nucleus of mature spermatozoa (Mali *et al.*, 1988). Nuclear DNA in the mature spermatozoa is thus packaged in a condensed form and the nucleus is transcriptionally inactive (Hecht, 1990). At this stage of development the cytoplasmic volume of the spermatid is reduced by the elimination of water. This is accomplished by the formation of specialised structures between the Sertoli cells and the head region of late spermatids which are known as tubulobulbar complexes (Russell, 1979). At the time of release of the mature spermatozoa from the Sertoli cell this cytoplasm retained by the tubulobulbar complexes and the resulting "residual body" is ultimately phagocytosed by the Sertoli cell.

2.2.1.2 The blood testis barrier

The presence of a permeability barrier surrounding seminiferous tubules of the mammalian testis has been well documented both by physiological (Vitale *et al.*, 1973) and morphological studies (Dym & Fawcett, 1970). Testicular injection of tracers such as peroxidase and lanthanum revealed that in rats the "barrier" forms between 15 and 18 days postnatally (Vitale *et al.*, 1973). Inhibition of gonadotrophin release delays but does not prevent the formation of the barrier. When germ cells were depleted from seminiferous tubules by irradiation *in utero* it was observed that testicular ABP, which first appears in the epididymis via the seminiferous tubule lumen at the time of barrier formation, was not measured in the epididymis until 30 days postnatally (Tindall *et al.*, 1975). This indicates that germ cells are not essential for barrier formation but that as with the gonadotrophins their absence may lead to a delay in its formation.

The barrier is composed of two elements; the peritubular myoid cell layer surrounding the seminiferous epithelium and, more importantly, tight junctions formed basally between adjacent Sertoli cells (Russell, 1993a). The outer membranes of neighbouring Sertoli cells are fused and there are characteristic granules found in the membranes of the junctions (Fawcett *et al.*, 1970). This results in the formation of two main compartments in the seminiferous tubules; a basal compartment between the Sertoli cell tight junctions and the basal lamina which contains the spermatogonia, and an adluminal compartment above the junctions containing most of the spermatocytes and the round and elongating spermatids. Preleptotene and perhaps leptotene spermatocytes are contained within an intermediate compartment which is formed during the passage of the developing germ cells into the adluminal compartment of the tubule (Russell, 1993a). The barrier acts to enable Sertoli cells to create and maintain a unique environment for development of the germ cells. It is also necessary to sequester the haploid germ cells from immune surveillance. Sertoli cells secrete fluid apically into the lumen of the tubules which is necessary to facilitate sperm transport from the testis (Setchell, 1969). The presence of junctions between the Sertoli cells is thought to be important in maintaining this fluid filled tubular lumen in the seminiferous tubule (Russell *et al.*, 1989).

2.2.1.3 Maturation and structure of the Sertoli cell

The Sertoli cell was first described by Enrico Sertoli in 1865 and it is thought to be the primary regulator of spermatogenesis. It is the only somatic component of the seminiferous epithelium and its function is known to change according to the stage of the spermatogenic cycle (Parvinen, 1993). The presence of the Sertoli cell barrier means the adluminal germ cells are dependent on factors produced by or passing through the cytoplasm of the Sertoli cells for their maturation. The Sertoli cell therefore provides a specialised environment for the growth and differentiation of germ cells.

Determination of Sertoli cell number. Sertoli cells are formed early on in foetal life and proliferation continues until about 2 weeks after birth in the rat (Orth, 1982). This proliferation appears to be under the control of FSH. In rats hemicastration results in a rise in FSH levels and a corresponding increase in ^{3}H-thymidine uptake by Sertoli cells in the remaining testes (Orth *et al.*, 1984). During early development thyroid hormone acting via receptors on Sertoli cells has also been shown to be involved in the control of Sertoli cell proliferation (Cooke & Meisami, 1991). Hypothyroidism induced in the rat from the day of birth to day 25 caused testicular enlargement in the adult due to an increase in Sertoli cell number which in turn was responsible for an increase in numbers of germ cells per testis (Hess *et al.*, 1993). The number of Sertoli cells produced during foetal and perinatal life has been shown to be critical in determining the level of sperm output in adult life. When Sertoli cell division was inhibited by injection of an anti-mitotic drug to rats immediately after birth the number of Sertoli cells in the adult was shown to be dramatically reduced compared to controls (Orth *et al.*, 1988). This decrease in Sertoli cell number was shown to be accompanied by an equivalent decrease in spermatid number. It is clear from this and other studies that the primary factor in determining daily sperm production in adult mammals is the number of Sertoli cells since each Sertoli cell can support only a finite number of germ cells (Russell & Peterson, 1984).

Morphology of the Sertoli cell. During postnatal and pubertal development the number of germ cells in the testis increases dramatically. The morphology of the Sertoli cell is observed to change at this time possibly to meet the demands of the developing germ cells (Chemes *et al.*, 1979). The Sertoli cells enlarge and develop cytoplasmic projections that extend between spermatogonia, spermatocytes and spermatids. The adult Sertoli cell is a columnar cell that extends from the base of the seminiferous epithelium to the lumen of the tubule (Russell, 1993b). It has a large and irregular nucleus situated in the basal part of the cell near the interstitial compartment. The cytoplasm contains an extensive network of endoplasmic reticulum and Golgi apparatus indicating that the cell is actively involved in protein secretion. Also present in the cytoplasm are cytoskeletal elements, lipid droplets, glycogen granules, lysosomes and numerous mitochondria (de Kretser & Kerr, 1988). Morphological observations on the Sertoli cell have indicated the presence of a Sertoli cell "cycle" (Morales & Clermont, 1993). The volume of Sertoli cells has been shown to change at each stage of the spermatogenic cycle with stages VII-VIII and stages XII-XIV having the smallest and largest volumes, respectively. The volume of endoplasmic reticulum in the Sertoli cell varies according to the stage of the spermatogenic cycle which suggests that the synthesis and/or secretion of proteins by these cells is cyclic in nature (see section 2.2.1.5). Localisation of the Golgi apparatus and the number of lipid droplets and lysosomes in the Sertoli cell cytoplasm have also been shown to change according to the stage of the spermatogenic cycle (Ueno & Mori, 1990). The observation of morphological changes demonstrating a structural cycle of Sertoli cells is also suggestive of a functional cycle of these cells. This cyclicity of function is discussed in section 2.2.1.5.

2.2.1.4 Function of the Sertoli cell

The principle function of the Sertoli cell is to support spermatogenesis by providing germ cells with the factors necessary for their development. Some of these secretory products are reviewed in greater detail below (section 2.2.1.5).

The Sertoli cell is also thought to be responsible for the production of seminiferous tubule fluid (Setchell, 1969). This fluid is involved in the apical transport of Sertoli cell secreted proteins towards germ cells and the lumen of the tubule. It is also important for the transport of testicular spermatozoa from the testis to the epididymis. Tubule fluid has a potassium concentration greater than that found in blood (for review, see Waites & Gladwell, 1982). The total concentration of protein is lower and the constituent proteins, carbohydrates and steroid hormones differ from that found in plasma. These differences are thought to be due to, and maintained by, the presence of the blood-testis barrier. In the rat, production of seminiferous tubule fluid is first detectable at 20 days of age (Russell *et al.*, 1989). Its production appears to be under hormonal control with FSH stimulating production in the immature rat and testosterone being stimulatory in the adult (Jégou *et al.*, 1982; 1983).

Sertoli cells are active phagocytes (Clermont *et al.*, 1987). At stages immediately prior to their release, mature spermatids are attached to the Sertoli cell by the specialised tubulobulbar complexes (Russell, 1993a). These structures are involved in retaining excess spermatid cytoplasm as the residual body previously described in section 2.2.1.1 (see also section 2.4.1). After spermatid release the residual bodies are retained in the adluminal compartment of the seminiferous tubule (Russell, 1993c). At this stage the membranes of the residual bodies and the Sertoli cell are distinct but as these structures migrate to the base of the seminiferous epithelium the residual body plasma membrane dissolves and fusion with Sertoli cell lysosomes occurs. The contents of the residual bodies are then degraded by lysosomal enzymes (Morales *et al.*, 1985).

2.2.1.5 Sertoli cell secreted proteins

Sertoli cells are known to synthesise and secrete over one hundred proteins (Wright *et al.*, 1983; Sharpe *et al.*, 1993b). The pattern of secretion of these proteins is often observed to change in a cyclical manner according to the stage of the seminiferous epithelium (Parvinen, 1993). In the rat the amount of secreted proteins as measured by incorporation of radiolabelled methionine into proteins secreted by isolated staged seminiferous tubules is almost doubled at stages VI-VIII compared with earlier and later stages of the cycle (Sharpe *et al.*, 1993a). This increase in protein secretion appears to be regulated by testosterone as it does not occur in the absence of androgen. The secretion of proteins by Sertoli cells is bi-directional occurring both via the apex of the cell into the tubule fluid and via the base of the cell into interstitial fluid (Sharpe, 1988). The direction of secretion is probably determined by the action of the different proteins. Thus, apical secretion is directed towards the germ cells and proteins secreted predominantly in this direction will most likely have a role to play in germ cell development. Basally secreted proteins can modulate the function of Leydig cells or other cell types in the interstitium (Papadopoulos *et al.*, 1987). Determination of the route of protein secretion *in vitro* has recently been investigated using a bicameral chamber culture system (Onoda *et al.*, 1990).

The proteins and other factors secreted by the Sertoli cell are essential for the support of germ cell development. Germ cells are dependent on a supply of lactate from the Sertoli cells for energy metabolism as they cannot utilise glucose directly (Jutte *et al.*, 1982). Sertoli cells secrete many proteins which are identical or homologous to those found in serum such as the metal ion transport proteins transferrin and ceruloplasmin (Skinner & Griswold, 1980; 1983). They produce numerous proteases including plasminogen activator and cathepsin L which may be involved in tissue remodelling during germ cell movement in the seminiferous epithelium and growth factors such as TGFß and IGF-1 which may be involved in germ cell proliferation and development (Parvinen, 1993). Although our knowledge of products that the Sertoli cell is capable of secreting is growing rapidly, exactly how the Sertoli cells support spermatogenesis is still poorly understood. However, it is generally accepted that germ cells rely on Sertoli cells for all their requirements for growth and differentiation. This is illustrated by the poor survival of spermatogenic cells in culture in the absence of Sertoli cells (Tres & Kierszenbaum, 1983). Whilst it would be impossible to mention all the known products of the Sertoli cell a few selected proteins are described in detail below and where possible their potential role in the support of spermatogenesis is discussed.

Transferrin Transferrin is an iron binding protein and is necessary for the transport of iron to cells where it is utilised. Synthesis of a transferrin-like protein by Sertoli cells in culture was first shown by Skinner & Griswold in 1980. FSH, insulin, retinol and testosterone (FIRT) were shown to stimulate transferrin synthesis *in vitro* by Sertoli cells isolated from rats aged 20 days either alone or in combination with each other (Skinner *et al.*, 1989a). Transferrin mRNA has been localised to Sertoli cells by *in situ* hybridisation (Morales *et al.*, 1987) and has also been demonstrated to be positively regulated by FIRT (Huggenvik *et al.*, 1987). In 40 day old rats which were hypophysectomised at 20 days of age, the level of transferrin mRNA was dramatically reduced compared to sham operated controls (Hugly *et al.*, 1988). This decrease could be partially prevented by treatment with FSH but not with testosterone. However, in rats hypophysectomised at 40 days of age testosterone was more effective in preventing the decrease in transferrin mRNA. These results are consistent with the changing influence of hormones on spermatogenesis with age (Steinberger *et al.*, 1978; Means *et al.*, 1980; Jégou *et al.*, 1982; Danzo *et al.*, 1990).

The level of transferrin produced by the Sertoli cell has been shown to change according to the stage of the spermatogenic cycle. Secretion of transferrin by freshly microdissected seminiferous tubules was maximal at stages IX-XIII and lowest in earlier staged tubules (Mather *et al.*, 1983). This stage specific production of transferrin appears to be regulated by germ cells and will be discussed in section 2.4.1. A model has been proposed for transferrin action in the testis (Huggenvik *et al.*, 1984). The initial step involves binding of diferric serum transferrin to receptors on the basal side of the Sertoli cells. This is internalised by receptor mediated endocytosis where the iron then interacts with Sertoli cell transferrin and is transported adluminally for use by the germ cells. Transferrin receptors have been localised on spermatocytes and early spermatids providing evidence in support of this model (Sylvester & Griswold, 1984). Transferrin production by the Sertoli cell and its regulation are described in greater detail in chapter 5 of this thesis.

Androgen Binding Protein. Sertoli cells secrete an androgen binding protein (ABP) which binds testosterone and dihydrotestosterone (DHT) with high affinity (French & Ritzen, 1973). ABP is a dimer of 89kDa composed of subunits of 46kDa and 43kDa which are encoded by a single gene but show differential glycosylation. The protein has been localised by immunostaining to the Sertoli cells of the seminiferous epithelium and to epithelial cells of the epididymis (Attramadal *et al.*, 1981). ABP is secreted by Sertoli cells into the lumen of the seminiferous tubules from where it is transported to the epididymis and internalised by the epithelial cells. It can be detected in the rat testis as early as 14 days postnatally but can only be measured in the epididymis from about 20 days postnatally, about the time of lumen and "barrier" formation in the testis (Tindall & Means, 1976; Danzo *et al.*, 1990).

The secretion of ABP by the Sertoli cell is hormonally regulated. Intravenous injection of ovine FSH to 10 day and 14 day old rats caused an increase in ABP secretion as measured by DHT binding but a similar increase was not observed in adult rats (Tindall & Means, 1976). However, following hypophysectomy in the adult the level of ABP decreased and could be restored by administration of partially purified FSH. In contrast a highly purified batch of FSH failed to influence the level of secretion. ABP secretion by 14 day old rats was stimulated by an injection of low amounts of LH indicating that the effect of the impure FSH sample was due to LH contamination. This effect was found to be via the Leydig cells due to LH stimulation of testosterone production. The importance of testosterone in the regulation of ABP secretion *in vivo* has been confirmed in studies using a GnRH antagonist (Danzo *et al.*, 1990). These authors showed that in the presence of antagonist ABP production in 20- and 30-day old rats was increased dramatically by hCG or testosterone but that FSH had only a small effect. FSH and hCG were both shown to be necessary for increased transport of ABP to the epididymis of 20 day old rats while testosterone alone was ineffective. Seminiferous tubule fluid is the only route of transport to the epididymis and therefore is responsible for the transport of ABP in young animals. This is consistent with data on detection of ABP in the epididymis as production of this fluid is

FSH regulated in immature animals and is also dependent on formation of the Sertoli cell barrier (Jégou *et al.*, 1982; Russell *et al.*, 1989).

The cDNA for rat ABP was isolated from a rat testis cDNA library and has been used as a probe to study the regulation of ABP mRNA (Joseph *et al.*, 1985). The cDNA was shown to hybridise to one major transcript of 1.7kb and a minor transcript of 2.3kb which may be produced due to differential splicing or polyadenylation (Hall *et al.*, 1990). The amount of mRNA per Sertoli cell increases to reach a maximum 20 days after birth and thereafter remains relatively constant. Using Sertoli cells cultured from immature rats the level of ABP mRNA was higher in cells cultured with FSH compared to those cultured in its absence (Hall *et al.*, 1990). This modulation appears to be mediated by cAMP as addition of dibutryl cAMP to the cultures increased ABP mRNA. The level of ABP mRNA in cultured Sertoli cells from 15 day old rats is also stimulated by treatment with a combination of FSH, insulin and retinol (Norton & Skinner, 1989).

Rat testicular ABP is homologous to sex hormone-binding globulin (SHBG) which is produced by the liver and is the major carrier of androgens (and oestrogens) in the blood of many species with the exception of the rat but including man (Joseph *et al.*, 1987). These proteins have been found to be encoded for by the same gene (Hammond *et al.*, 1989) but they differ in their pattern of glycosylation. This glycosylation does not appear to affect steroid binding but it may be important for the secretion of ABP from the Sertoli cell (Joseph *et al.*, 1992). The exact role of ABP in the testis remains unclear but it may be involved in the regulation of androgen action on spermatogenesis. A recent study has demonstrated the possible presence of a receptor on isolated rat germ cells which specifically binds ABP and hSHBG (Felden *et al.*, 1992). It is proposed that a receptor-ABP-androgen complex may be internalised by receptor mediated endocytosis as has been shown for hSHBG in monkey germ cells (Gérard *et al.*, 1992). However, elucidation of the role of ABP in the testis requires further study.

The sulphated glycoproteins. The sulphated glycoproteins SGP-1 and SGP-2 are major components of the total protein secreted by Sertoli cells. They both exhibit extensive charge heterogeneity when analysed on two dimensional electrophoresis gels, consistent with the presence of several differentially glycosylated forms.

SGP-1 is a monomeric protein of 70kDa which is heavily glycosylated and sulphated. SGP-1 mRNA and its protein product have been localised to the Sertoli cells of the seminiferous epithelium by *in situ* hybridisation and indirect immunofluorescence, respectively (Collard *et al.*, 1988). The protein was localised more specifically to the lysosomes of the Sertoli cells and to residual bodies but only after fusion with lysosomes at stage IX of the spermatogenic cycle (Sylvester *et al.*, 1989). Significant sequence homology at both the DNA and amino acid level was found to the precursor of human sphingolipid activator protein (also known as the sulphatide/ G_{M1} activator protein or prosaposin; Collard *et al.*, 1988). Prosaposin is proteolytically cleaved into smaller proteins called the saposins which are normally present in lysosomes and act to solubilise lipids ready for hydrolysis (O'Brien *et al.*, 1988). The presence of SGP-1 in lysosomes following fusion with residual bodies suggests a role for the protein in the degradation of glycolipids in residual bodies (Sylvester *et al.*, 1989). The action of secreted SGP-1 is less clear but it may be involved in lipid metabolism or transport between Sertoli cells and germ cells or within the reproductive tract (Collard *et al.*, 1988).

SGP-2 synthesised by Sertoli cells has been localised by indirect immunofluorescence to Sertoli cell cytoplasm, cells of the epididymis and to the acrosome, neck and endpiece of mature sperm (Sylvester *et al.*, 1984). The protein is synthesised as a 73kDa glycosylated precursor molecule which is then cleaved to give the mature form, a dimer composed of subunits of 47kDa and 34kDa covalently linked by disulphide bonds. Its cDNA has been cloned (Collard & Griswold, 1987) and the mRNA is detectable in Sertoli and epididymal cells from 7 day old rats but increases dramatically in 14 day old rats after which the level remains relatively constant (Zakeri *et al.*, 1992). Analysis of the mRNA content of various tissues show a wide distribution of SGP-2 with highest levels occurring in Sertoli cells and the epididymis although SGP-2 synthesised by the latter has a different pattern of glycosylation to the testicular form. It has been proposed that SGP-2 coats the mature sperm as it leaves the testis but is stripped off as it passes through the rete testis and efferent ducts and is endocytosed by cells lining these regions (Hermo *et al.*, 1991). Thereafter sperm are recoated by SGP-2 secreted in the epididymis. The functional significance of this is unknown. The protein has been localised by immunostaining specifically to the lysosomes of Sertoli cells at all stages of the spermatogenic cycle and in residual bodies at stages IX-X after fusion of these structures with the lysosomes (Sylvester *et al.*, 1991). The role of SGP-2 in the testis and epididymis remains to be elucidated.

Within the seminiferous epithelium, SGP-2 is synthesised at all stages of the spermatogenic cycle with maximal secretion occurring at stages VII-VIII (Kangasniemi *et al.*, 1992). Hypophysectomy of 20 day and 40 day rats leads to a reduction in the level of SGP-2 mRNA (Hugly *et al.*, 1988). This decrease can be partially prevented by injection of FSH to 20 day old rats and administration of testosterone in the older rats. However, SGP-2 mRNA expression is not affected by hypophysectomy in adult rats or by subsequent treatment with testosterone (Roberts *et al.*, 1991). *In vitro* the addition of FSH to adult staged seminiferous tubules has no effect on the secretion of SGP-2 (Kangasniemi *et al.*, 1992). Taken together these data suggest that either a change in the regulation of SGP-2 production is occurring as the animals grow older or it may reflect an overall change in the responsiveness of the Sertoli cell to these hormones.

Several proteins have been identified which show sequence homology to SGP-2. At the nucleotide level it has homology with human proapolipoprotein A-I precursor and it has been suggested that SGP-2 may be involved in lipid transport (Collard & Griswold, 1987). It has also been shown to be identical to a protein known as clusterin which was isolated independently from ram rete testis fluid and caused cell aggregation in suspensions of Sertoli cells, erythrocytes and mouse TM-4 cells (a cell line thought to be derived from immature Sertoli cells; Fritz *et al.*, 1983). SGP-2 has been separately identified as testosterone-repressed prostate message-2 (TRPM-2) and appears to be expressed in cells undergoing programmed cell death (Buttyan *et al.*, 1989). The forms of SGP-2 expressed in regressing prostate differ in molecular mass from testicular SGP-2. This may simply be due to differential processing in the two tissues or may indicate that the proteins made during cell death serve a different function from those in the normal male reproductive tract. Yet another homologue of SGP-2 is the human protein SP-40,40 which is involved in the suppression of complement mediated cell lysis (Kirszbaum *et al.*, 1988). Structural analysis of SGP-2 shows several areas which would enable the protein to interact with lipids and amphiphatic domains in other proteins. However, although SGP-2 has been localised and assigned roles in many tissues its exact function in the testis requires further investigation.

Inhibin. Inhibin is a dimeric glycoprotein which can inhibit secretion of FSH from the pituitary gland (for review see de Jong, 1988). It is composed of an α subunit and one of two forms of ß subunit (ßA or ßB) covalently linked by disulphide bonds. Immunostaining has localised the inhibin α-subunit and the ßB-subunit to Sertoli cells while the ßA-subunit was not detected (Shaha *et al.*, 1989). Messenger RNAs for α and ß subunits have also been identified in Sertoli cells (Roberts *et al.*, 1989). The level of inhibin measured by an RIA specific for the α-subunit was highest in plasma of 8-15 day old rats but thereafter was seen to decrease with age (Rivier *et al.*, 1988). Immunostaining confirmed that a decline in Sertoli cell inhibin content occurred with age, with very little positive staining observed in 90d old rats. Neutralisation of endogenous inhibin with antiserum increased plasma FSH levels in 10 day old rats but had no effect in rats older than 26 days (Rivier *et al.*, 1988). These results would suggest that inhibin is only important in regulating FSH production in the immature animal. However, studies involving germ cell depletion in the adult rat have shown that absence of elongate spermatids, which has been correlated to a decrease in inhibin secretion (Allenby *et al.*, 1991), coincides with an increase in serum FSH levels (Pinon-Lataillade *et al.*, 1988). These data would seem to suggest that inhibin may also have a role to play in controlling FSH secretion in the adult.

The addition of FSH to purified Sertoli cells from 22 day old rats in culture was shown to significantly increase secretion of immunoreactive inhibin (Toebosch *et al.*, 1989). The level of α-subunit but not ßB-subunit mRNA was also increased. Addition of testosterone to these cultures had no effect on inhibin levels. Hypophysectomy of both sexually immature and adult rats caused a decrease in inhibin α-subunit mRNA but had no effect on ßB-subunit mRNA (Krummen *et al.*, 1989). This decrease could be prevented or reversed by treatment with FSH. These studies show that FSH is an important pretranslational regulator of inhibin production, exerting an effect either at the level of transcription or mRNA stability.

The expression of α- and ßB-subunit mRNAs is dependent on the stage of the spermatogenic cycle (Bhasin *et al.*, 1989). Highest levels of both messages is seen at stages XIII-I and lowest levels at stages VII-VIII. These stages of maximal expression also correspond to stages of high FSH binding and FSH-stimulated cAMP production. The stage dependent expression of inhibin indicates that it may have a paracrine role to play in the testis in addition to an endocrine role in regulating FSH secretion. Further evidence for paracrine action comes from the demonstration of fluorescent labelled inhibin binding to germ cells separated by flow cytometry (Woodruff *et al.*, 1992). It has also been reported that inhibin injection causes a decrease in the number of spermatogonia in the testes of mice and hamsters *in vivo* (van Dissel-Emiliani *et al.*, 1989). The significance of these results in relation to the roles of inhibin in the testis is as yet unclear.

Growth factors. Sertoli cells are known to secrete a range of growth factors the exact function of which remains to be clarified. Interleukin-1α is produced in the testis and its principal site of synthesis is thought to be the Sertoli cell (Gérard *et al.*, 1991). The activity of this cytokine in Sertoli cell conditioned media on the proliferation of murine thymocytes in culture was blocked by anti-IL-1α but not by anti-IL-1ß demonstrating that Sertoli cells produce specifically IL-1α. DNA synthesis in A type spermatogonia and preleptotene spermatocytes occurs at specific stages of the spermatogenic cycle with two peaks at stages V and VIII (Parvinen *et al.*, 1991). It has been shown that microdissected stage VII tubules, which are not active in DNA synthesis, progress *in vitro* to stage VIII, initiate DNA synthesis and at the same time begin to secrete IL-1α. Stimulation of DNA synthesis in spermatogonia of stage I tubules was also found on addition of IL-1α. It is possible that stimulation of DNA synthesis by germ cells *in vivo* could be initiated by Sertoli cell derived IL-1α but interaction with several other growth factors is likely.

Messenger RNA for transforming growth factor-β_1 (TGFβ_1) has been identified in Sertoli cells (and peritubular cells) in the rat (Skinner & Moses, 1989). Proteins secreted from Sertoli cells of 20 day old rats were found to include one or more which bound to TGFβ receptors, and which cross reacted with antibodies for TGFβ_1 and TGFβ_2. Purified TGFβ_1 did not appear to influence the morphology or the function of Sertoli cells (as measured by secretion of transferrin) isolated from 20 day old rats when added to the cells in culture. *In vitro* protein secretion by peritubular cells in culture was influenced by addition of purified TGFβ_1 (Skinner & Moses, 1989). The cells showed an increase in the secretion of specific proteins corresponding to molecular weights for known extracellular matrix proteins. Migration of peritubular cells was also influenced and this could possibly be via effects on extracellular matrix proteins. However, the precise role of the various forms of TGFβ in testicular function and their action on Sertoli cells from immature or adult animals remains to be investigated.

The Sertoli cell has been shown to secrete several other growth factors such as transforming growth factor-α (Skinner *et al.*, 1989b), Sertoli cell secreted growth factor (Shubhada *et al.*, 1993) and insulin-like growth factor 1 (Bellvé & Zheng, 1989). However, the role these factors have to play in the paracrine modulation of testicular function is still to be elucidated and they will not be discussed in this review.

2.2.2 The interstitial compartment

2.2.2.1 Leydig cells

Leydig cell development. The embryonic origins of Leydig cells in the testis are unknown. The prepubertal rise in Leydig cell number occurring from approximately 14-28 days postpartum in the rat is due to division of precursor mesenchymal cells in the interstitial compartment of the testis (Hardy *et al.*, 1989). After this period of recruitment of mesenchymal cells the number of Leydig cells continues to rise to its adult level by division of the newly generated Leydig cells. FSH treatment of immature hypophysectomised rats was shown to stimulate Leydig cell maturation (Kerr & Sharpe, 1985). Treatment resulted in Leydig cell hypertrophy, the development of morphological features identical to those of adult Leydig cells and enhancement of hCG stimulated testosterone synthesis *in vitro*. LH had no effect on Leydig cell development. However, LH has been shown to cause a small increase in the incorporation of ^{3}H-thymidine into immature Leydig cells in culture (Khan *et al.*, 1992). Stimulation of DNA synthesis in Leydig cells was also increased by addition of the growth factors TGFα, TGFß and IGF-1 all of which may be derived from Sertoli cells as discussed in the previous section (2.2.1.5). The mechanisms by which these growth factors interact to stimulate DNA synthesis in Leydig cells *in vivo* is unknown.

Steroidogenic function of Leydig cells. Leydig cells are the site of synthesis of testosterone in the testis and this production is stimulated by LH produced by the pituitary gland (see section 2.3.5.2). LH receptors are first detected in foetal testis at day 15 of gestation, their numbers increase sharply at day 18 and reach a maximal level just before birth (Gangnerau *et al.*, 1982). An increase in testosterone content is observed at the time of this rise in LH receptor numbers but intratesticular testosterone content decreases after birth (Warren *et al.*, 1984). This decrease is not due to a decline in LH receptor number but instead may be due to the use of alternative steroidogenic pathways such as the production of 5-α-reduced androgens which is associated with an increase in the activity of 5-α-reductase at this time (Preslock, 1980).

Adult Leydig cells are identified by their characteristic polyhedral shape with an oval nucleus and a prominent nucleolus (de Kretser & Kerr, 1988). They contain many cytoplasmic inclusions including very large amounts of smooth endoplasmic reticulum indicative of a cell type active in steroid synthesis. Cholesterol is the precursor for steroid biosynthesis in the testis (Fig.3). This is obtained by *de novo* synthesis or is diverted from the plasma membrane to the mitochondria (for review see Gower, 1988). Free cholesterol is transported to the outer mitochondrial membrane probably bound to a carrier protein such as sterol carrier protein 2 (SCP_2; van Noort, 1986). This carrier protein is found only in the Leydig cells of the testis. Synthesis of SCP_2 is stimulated by hCG suggesting that its production may be important in regulating the availability of cholesterol for steroidogenesis. Cholesterol bound to its carrier protein must move to the inner mitochondrial membrane where it is converted to pregnenolone by the side-chain cleavage complex. Thereafter, pregnenolone is converted to testosterone in the smooth endoplasmic reticulum (Fig.3).

Testosterone biosynthesis is under the control of LH secreted by the pituitary gland as discussed in section 2.3.5.2. However, androgen synthesis can also be influenced by local factors produced by the seminiferous tubules (see section 2.4.2 for more detail) and by Leydig cells themselves. Autoregulation of testosterone production

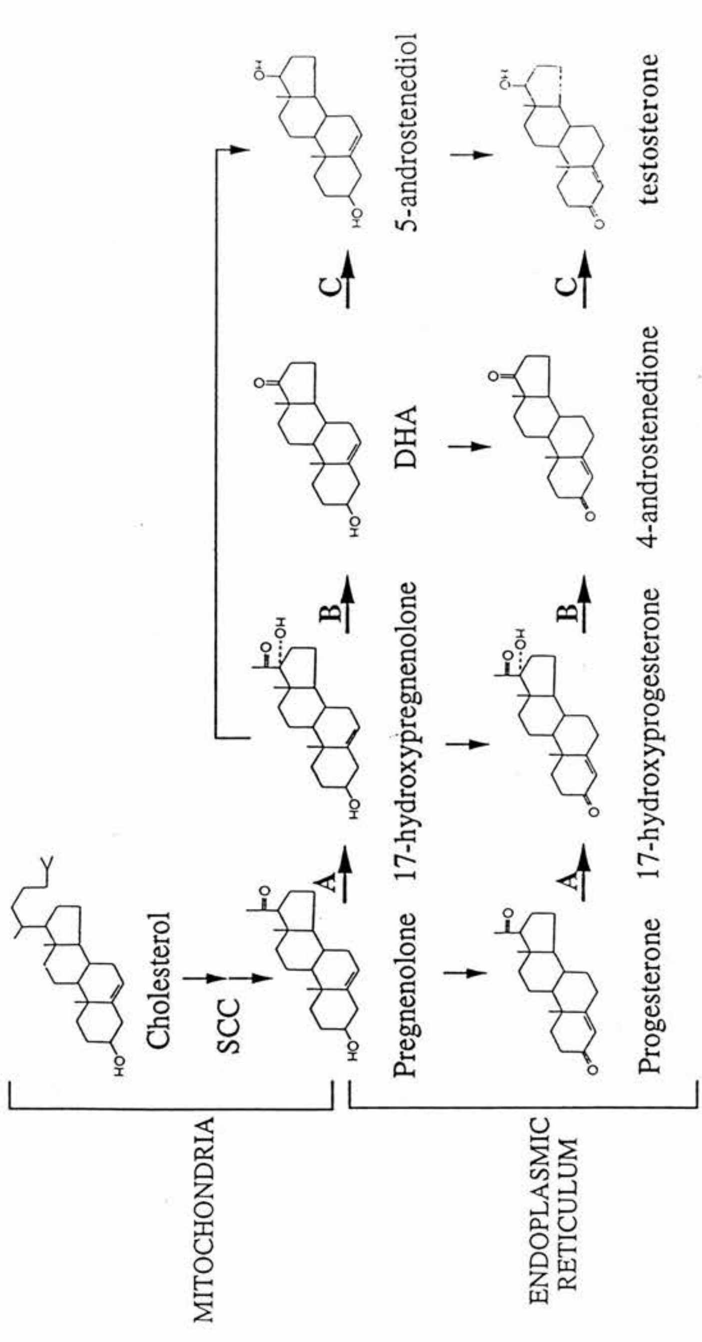


Figure 3. Androgen biosynthesis in Leydig cells. In man, the major route of testosterone synthesis is via the Δ^5-pathway including pregnenolone, 17-hydroxypregnenolone, dehydroepiandrosterone (DHA) and androstenediol. In other species including the rat the Δ^4-pathway is utilised via progesterone, 17-hydroxyprogesterone and androstenedione. The enzymes in the pathway are SCC, the cholesterol side chain cleavage complex located in the mitochondria; A, 17-dehydroxylase; B, C-17, 20-lyase; C, 17ß-hydroxysteroiddehydrogenase. A, B and C are all located in the endoplasmic reticulum of the Leydig cells. *Adapted from Gower, 1988.*

has been shown in the rat and rabbit (Darney & Ewing, 1981). Perifusion of testes by testosterone was observed to decrease production of testosterone by approximately 70% compared to controls and this regulation is specific to testosterone as there was no effect of other steroids, eg. corticosterone and androstenedione. Expression of androgen receptor mRNA has been shown in human Leydig cells as has high affinity binding of the tritiated steroid R1881 indicating that this autoregulation of steroid production may also occur in humans (Namiki *et al.*, 1991). The activity of aromatase, which is responsible for the conversion of androgens to oestrogens, increases in the Leydig cell during puberty (Rommerts *et al.*, 1982). Oestrogen produced in the adult rat has been shown to decrease Leydig cell responsiveness to stimulation with LH *in vitro* (Dorrington *et al.*, 1978). However, it is thought that the levels of oestrogen needed to impair steroidogenesis directly would never be reached *in vivo* and therefore oestrogen probably works indirectly by suppression of LH secretion at the pituitary level (Benahmed *et al.*, 1982). Interestingly, oestrogen has been shown to prevent the proliferation of Leydig precursor cells after destruction of adult Leydig cells by treatment with EDS (Abney & Myers, 1991). This would suggest that this hormone may be involved in the control of Leydig cell numbers under normal conditions.

POMC in the Leydig cell. Leydig cells in the adult rat testis have been shown to express pro-opiomelanocortin (POMC) mRNA (Pintar *et al.*, 1984). POMC mRNA has also been identified in some spermatogonia and spermatocytes of adult mice but the importance of this finding is not known. Immunostaining for the POMC-derived peptides adrenocorticotrophic hormone (ACTH), melanocyte stimulating hormone (MSH) and ß-endorphin has shown their presence in the Leydig cells of the testis of the rat and several other species (Bardin *et al.*, 1987).

The regulation of POMC expression in the testis appears to involve endocrine and paracrine interactions. In cultured foetal Leydig cells production of ß-endorphin was shown to be stimulated by hCG (Fabbri *et al.*, 1988). Total testicular content of POMC-derived peptides declines after hypophysectomy. These changes are reflected at the level of the mRNA. Interestingly, POMC mRNA does not appear to be expressed in all Leydig cells but was specific for those located in the interstitial space adjacent to seminiferous tubules at stages IX-XII of the spermatogenic cycle (Gizang-Ginsberg & Wolgemuth, 1985). This suggests an influence of seminiferous tubules on Leydig cell function.

The function of POMC-derived peptides in the testis is uncertain. ACTH and MSH have been shown to stimulate proliferation of immature Sertoli cells in culture (Bardin *et al.*, 1987). Accumulation of cAMP and an increase in adenylate cyclase activity was also demonstrated. In contrast, injection of opioid receptor antagonists such as naloxone into the testis of neonatal rats has been shown to cause testicular hypertrophy suggesting that endogenous ß-endorphin may normally be involved in inhibiting Sertoli cell proliferation (Orth, 1986). No such effect is seen in adult rats but naloxone treatment did cause a decrease in basal and hCG stimulated testosterone secretion by Leydig cells *in vitro* (Gerendai *et al.*, 1984). These results would suggest that POMC-derived peptides are involved in regulation of testicular function but that their site of synthesis and mode of action *in vivo* remains unclear.

Inhibin and activin in Leydig cells Inhibin and activin are gonadal peptides involved in the negative and positive regulation of FSH production by the pituitary gland, respectively (Scott *et al.*, 1981; Ling *et al.*, 1986a; 1986b). Whether bioactive inhibin is produced by Leydig cells is still being debated. Several reports have shown the secretion of inhibin α-subunit by Leydig cells in culture (eg. Risbridger *et al.*, 1989) and α-inhibin mRNA has been detected in these cells using Northern blot analysis (de Winter *et al.*, 1992). In contrast, an immunolocalisation study has detected ßA and ßB but not α subunit protein in both immature and adult rat Leydig cells (Ogawa *et al.*, 1991). It is not thought that Leydig cells can secrete bioactive inhibin (Krummen *et al.*, 1992) although this has been claimed in some studies (Risbridger *et al.*, 1989). Leydig cells from immature rats also appear to possess receptors for activin and inhibin (Krummen *et al.*, 1992). Activin bioactivity has been observed in interstitial cells of immature rat testis but not in adult Leydig cells and it has been shown to stimulate testosterone release from purified rat Leydig cells in culture (Lin *et al.*, 1989). However, using cultured mixed testicular cells, activin decreased while inhibin increased LH-stimulated testosterone production (Hsueh *et al.*, 1987). In contrast, data from an *in vivo* study in the adult macaque failed to show an effect of activin on testosterone production while a significant stimulation of FSH secretion was demonstrated (McLachlan *et al.*, 1989). It is clear from these studies that the actions of these gonadal peptides on Leydig cells can be influenced by the presence of other cell types in the testis, may be dependent on the age of the animal and may be species specific. The exact site/s of production and function of these peptides in the testis still remains to be determined.

Growth factors and the Leydig cell. Transforming growth factor α (TGFα) has been localised by immunostaining to Leydig cells at all stages of development (Teerds *et al.*, 1990). It is not yet known if TGFα is synthesised in Leydig cells since to date its mRNA has only been found in seminiferous tubules (Skinner *et al.*, 1989b) but it is likely that these cells also express the mRNA. This growth factor shows sequence homology to epidermal growth factor (EGF) and can bind to the EGF receptor with high affinity. The protein is synthesised as a precursor molecule with a transmembrane domain which enables it to anchor in the plasma membrane. The mature form of TGFα is the extracellular domain which is cleaved off the membrane and can act on cells distant from its site of synthesis. However, the growth factor is also active while anchored in the plasma membrane and has been shown to act on neighbouring cells. It has been proposed that TGFα has a role in the regulation of Leydig cell growth and differentiation (Khan *et al.*, 1992).

Immunoreactive basic fibroblast growth factor (bFGF) has been identified in Leydig cells during embryonic development but in the adult rat testis is found mainly in germ cells (Mather & Krummen, 1992). Testosterone secretion by immature rat Leydig cells in culture was shown to be inhibited by bFGF acting on 17α hydroxylase in the steroidogenic pathway (Fauser *et al.*, 1988) whilst bFGF stimulated basal and hCG-stimulated testosterone production in immature porcine Leydig cells (Sordoillet *et al.*, 1988). It appears that bFGF is involved in the regulation of testosterone production probably via the control of steroidogenic enzyme activity in the maturing Leydig cell but its role, if any, in the adult remains unknown.

2.2.2.2 Macrophages

In the normal adult testis macrophages make up 14-16% of all cells in the interstitial compartment. In testes showing tubular damage due to cryptorchidism or irradiation the number of macrophages in the interstitium increases significantly (Niemi *et al.*, 1986). These cells are active phagocytes and are the site of synthesis of several cytokines such as tumour necrosis factor (TNF) and interleukin-1ß (IL-1ß). In cultures of dispersed whole testis cells both TNF and IL-1ß are found to increase testosterone production (Warren *et al.*, 1990). However, since the stimulation of testosterone production in purified Leydig cells was not as great as with a mixed cell population it would appear that the cytokines do not act directly on Leydig cells but that the effect is mediated via another cell type. In other reports, IL-1ß inhibited Leydig cell steroidogenesis in culture (Calkins *et al.*, 1988). Although it is unclear whether these factors stimulate or inhibit steroidogenesis these studies suggest that cytokines may be involved in gonadal function. TNF has also been shown to increase the secretion of transferrin from Sertoli cells isolated from stage IX-XI and stage XIII tubules in culture (Boockfor & Schwarz, 1991). These results suggest that macrophages may play a role in the paracrine regulation of testicular function but the importance of their role is unknown.

2.2.2.3 The vasculature

Blood vessels are an important component of the interstitial tissue of the mammalian testis. Data from recent studies are consistent with the vasculature having a role to play in controlling testicular function and its importance is suggested by the observation of a positive correlation between testicular blood flow and both testosterone secretion and testicular size (Setchell, 1990; Damber & Bergh, 1992a). In turn, testosterone can maintain testicular blood flow in testis depleted of Leydig cells due to ethane dimethanesulphonate (EDS) treatment (Damber *et al.*, 1992). Capillaries in the testis show differences in permeability compared to blood vessels in other organs; they are not permeable to dyes that would normally cross blood vessel walls but have a high permeability for other substances such as albumin (Damber & Bergh, 1992a). Blood flow in the capillaries of the testis shows rhythmical variations termed vasomotion. Destruction of Leydig cells by treatment with EDS inhibited vasomotion but it could be re-established by treatment with low doses of testosterone (Collin *et al.*, 1993). The recent demonstration of androgen receptors on blood vessels suggests testosterone may be capable of a direct effect on vasomotion through interaction with androgen receptors on the capillary walls (Damber & Bergh, 1992b).

2.3. Endocrine control of testicular function

2.3.1 GnRH production by the hypothalamus

The secretion of the pituitary gonadotrophins, FSH and LH, is under the control of gonadotrophin releasing hormone (GnRH) synthesised by the hypothalamus (Redding *et al.*, 1972; see Fig.4). In the rat or monkey, prevention of GnRH action on the pituitary, either physically by stalk section or functionally by the use of GnRH antibodies, results in inhibition of FSH and LH secretion and the arrest of gonadal function (Vaughan *et al.*, 1980; McCormack *et al.*, 1977; Ellis *et al.*, 1983). GnRH is released from the hypothalamus in a pulsatile manner and this pattern of release is essential for normal secretion of the pituitary gonadotrophins (Belchetz *et al.*, 1978). Testosterone is involved in the negative regulation of GnRH secretion being responsible in part for the slow frequency of its release (Plant *et al.*, 1978; Matsumoto & Bremner, 1984).

GnRH is a decapeptide with specific amino acids important for binding to its receptor and for receptor activation (Conn, 1986). A cDNA coding for the GnRH receptor has recently been cloned in the mouse and the predicted amino acid sequence was shown to be typical of a member of the seven transmembrane domain, G-protein

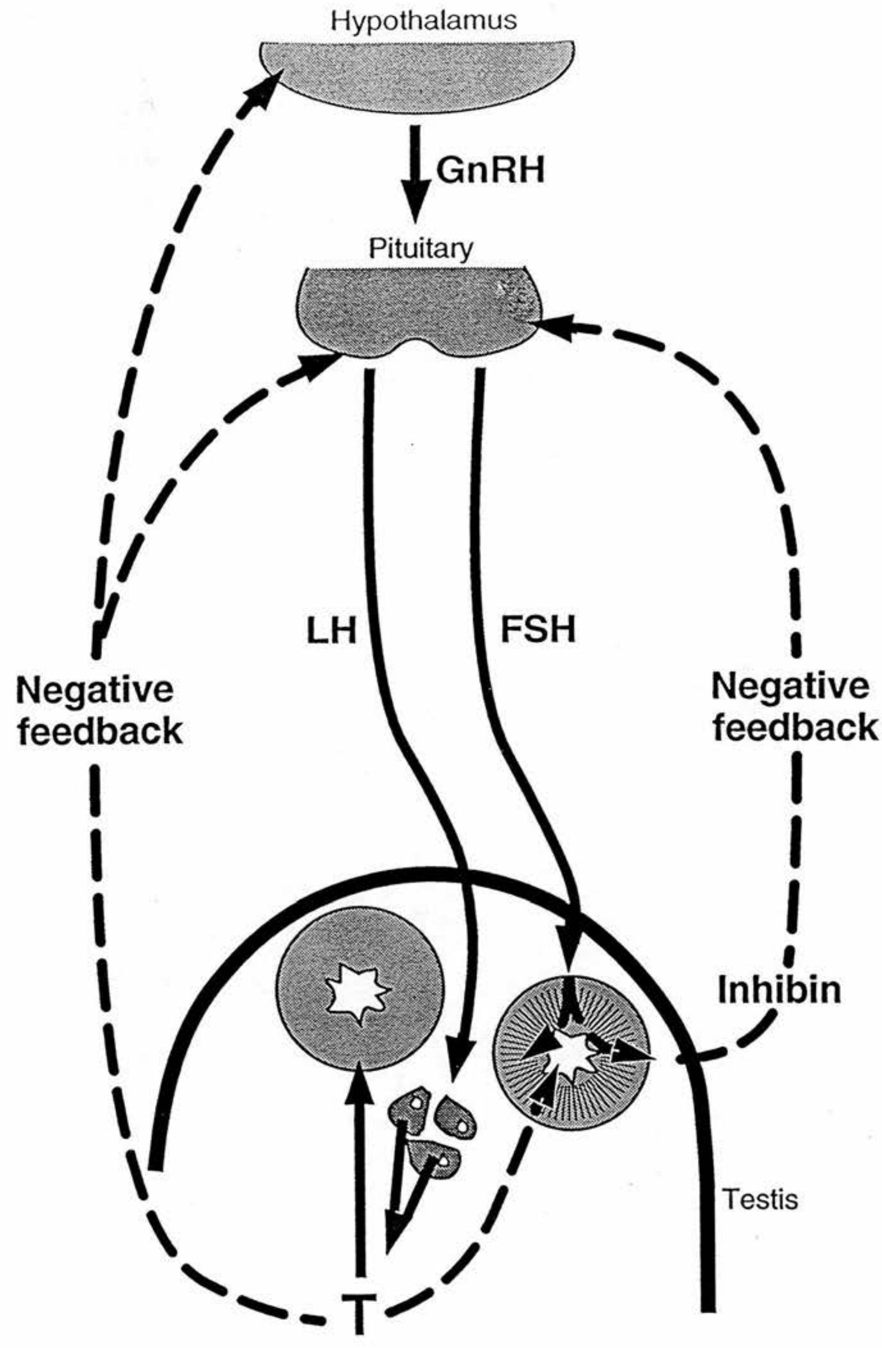


Figure 4. Endocrine control of testicular function. Shows the positive modulation of testicular function by hormones produced in the hypothalamus and the pituitary gland. Negative feedback from the testis is also indicated. GnRH, gonadotrophin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinising hormone; T, testosterone.

coupled receptor family (Tsutsumi *et al.*, 1992). Receptor numbers in the pituitary are regulated partly by GnRH itself and the concentration of receptors is directly related to the responsiveness of the pituitary gland (Clayton *et al.*, 1982). Ca^{2+} has been implicated as the primary second messenger involved in this system (Marian & Conn, 1979) but other signalling pathways involving phospholipase C, protein kinase C and diacylglycerols may also have a role to play in the pituitary response to GnRH (for review see Conn, 1986).

2.3.2 The pituitary gonadotrophins

FSH and LH are members of a family of glycoprotein hormones which also includes TSH secreted by the pituitary and in some species hCG produced by the placenta (for review see Pierce & Parsons, 1981). This family show many structural similarities being heterodimers consisting of an α subunit common to all the family members and a unique ß subunit which confers the biological specificity of the hormone. The α subunit consists of 96 amino acids when in its mature form and the ß subunits consist of 121 and 110 amino acids for LH and FSH, respectively (Counis & Jutisz, 1991). These subunits are found in a non-covalent association and are glycosylated at specific residues along their length. The carbohydrate groups are thought to be important for the biological activity of the hormones and the interaction with their receptors.

2.3.3 Regulation of gonadotrophin production

The production of FSH and LH is initiated by GnRH produced by the hypothalamus as described above. GnRH appears to act at the level of transcription as disconnection of the hypothalamus and pituitary gland, either functionally or physically, leads to a decrease in the expression of α, LHß and FSHß subunit mRNAs (Hamernik *et al.*, 1986). This decrease can be reversed or prevented by administration of pulsatile GnRH with the frequency and amplitude of the GnRH pulses affecting the amount of gonadotrophin mRNA (Haisenleder *et al.*, 1988; Rodin *et al.*, 1989).

The production of these hormones can also be influenced by several other factors both steroidal and non-steroidal. Pituitary tissue removed from castrated rats has been shown to contain greater levels of α, LHß and FSHß mRNA available for translation in cell-free systems compared to tissue from intact animals (Godine *et al.*, 1980). This regulation has been shown more directly using Northern blot analysis of pituitary mRNA in which mRNA levels were again increased after castration compared to controls with the rise in FSHß mRNA being more modest than that of the other two subunits (Gharib *et al.*, 1987; Abbott *et al.*, 1985). The number of gonadotrophs containing LH, shown by immunostaining, also increased after gonadectomy (Ibrahim *et al.*, 1986). Testosterone replacement in male rhesus monkeys has been shown to maintain serum gonadotrophins at normal levels following castration while withdrawal of this treatment leads to a post-castration rise in FSH and LH (Plant *et al.*, 1978). The levels of α and LHß mRNA levels in castrated male rats were depressed to almost normal values by testosterone treatment but there was no effect on FSHß mRNA levels (Wierman *et al.*, 1989). It has been shown more recently in transgenic mice carrying the gene for human FSHß that expression of FSHß mRNA can be suppressed by testosterone following castration of male mice (Kumar & Low, 1993). This would indicate that there may be important species differences in regulation of the gonadotrophins.

FSH production has been shown to be regulated by inhibins, produced by the gonads and by activins (see also sections 2.2.1.5 and 2.2.2.1; for review see Ying, 1988). However, to date there is no direct evidence that activin is actually synthesised in the gonads or can circulate in the blood to reach the pituitary gland, although synthesis by the pituitary gland has been shown (Ying, 1988). Inhibin has been shown to directly suppress the synthesis of FSH in the pituitary (Scott *et al.*, 1981) both by suppression of FSHß mRNA levels as well as translation of the mRNA (Mercer *et al.*, 1987). In contrast the activins appear to be involved in positively regulating FSH release from the pituitary gland (Ling *et al.*, 1986a; 1986b). LH release is not generally thought to be affected by the activins or inhibins although infusion of human recombinant activin-A into adult macaques has been shown to result in a significant increase in GnRH-stimulated, but not basal, LH secretion (McLachlan *et al.*, 1989).

2.3.4 The gonadotrophin receptors

The gonadotrophin receptors, luteinising hormone receptor (LH-R) and follicle-stimulating hormone receptor (FSH-R) are plasma membrane receptors and in the testis are expressed on the surface of Leydig and Sertoli cells, respectively. The cDNAs for LH-R and FSH-R have recently been cloned and the amino acid sequences predicted (McFarland *et al.*, 1989; Sprengel *et al.*, 1990). The data suggest they belong to a family of receptors including those for rhodopsin, serotonin, dopamine and acetylcholine which all show structural and functional similarities (Strosberg, 1991).

2.3.4.1 The luteinising hormone receptor

From the cDNA sequence, the LH-R was shown to be a single polypeptide of 674 amino acids with a predicted molecular mass of 75kDa. The N-terminal domain is proposed to exist extracellularly, contains several potential glycosylation sites and is responsible for the high affinity binding of the hormone to LH-R (McFarland *et al.*, 1989). It has been shown that glycosylation is not necessary for ligand binding but is required for activation of adenylate cyclase (Sairam, 1989). The extracellular domain is followed by a characteristic seven transmembrane domain and then a C-terminal domain which is thought to be located intracellularly. There are several serine, threonine and tyrosine residues in this region which are sites of potential phosphorylation and may be important for the function and/or regulation of the receptor. Receptor interaction with its G-protein/s on hormone binding is thought to result in activation of adenylate cyclase causing an increase in cAMP which is the principal mediator of the actions of LH in gonadal cells (Cooke, 1990).

Desensitisation of the gonadal cells to LH has been shown to occur and this can be due to either uncoupling or down-regulation of the LH-R. Uncoupling does not involve a change in receptor numbers but is a functional change in the receptor which does not allow activation of the effector system on binding of the ligand. It has been shown by mutagenesis studies that the last 43 amino acids in the cytoplasmic tail of the receptor are required for uncoupling to occur although the exact mechanism by which this occurs, eg. phosphorylation, is still unclear (Sanchéz-Yagüe *et al.*, 1992). Down-regulation of the response to LH occurs by a decrease in the number of receptors being expressed on the cell surface (Freeman & Ascoli, 1981). Studies using a mouse Leydig cell tumour cell line (MA-10) have shown that this occurs initially by increased degradation of the receptor protein but after prolonged exposure to LH the expression of LH-R mRNA also decreases (Wang *et al.*, 1991).

2.3.4.2 The follicle stimulating hormone receptor

The cDNA sequence of the FSH-R predicts a mature protein of 672 amino acids with a molecular weight of 75kDa and a structure similar to the LH-R (Sprengel *et al.*, 1990). The extracellular domains of the LH and FSH-receptors share 50% sequence homology while the sequences defining the seven transmembrane domains have 80% sequence similarity. The N-terminal domain contains eight cysteine residues which are conserved between the FSH-R, LH-R and TSH-R indicating that they may be important in maintaining the conformation of the large glycosylated extracellular domain (Sprengel *et al.*, 1990). FSH binding to its receptor causes an increase in cAMP and this is probably mediated by a G-protein and adenylate cyclase as with the LH-R. However unlike the LH-R, FSH receptors do not appear to be down regulated by high levels of the ligand (Yoon *et al.*, 1990).

2.3.5 Mechanism of action of the gonadotrophins

2.3.5.1 FSH action on the Sertoli cell

In the testis FSH has been shown to act primarily on Sertoli cells. In the presence of a phosphodiesterase inhibitor an increase in cAMP was detectable on addition of FSH to rat seminiferous tubules *in vitro* (Dorrington & Fritz, 1973). FSH was able to stimulate cAMP levels even after germ cell depletion due to hypophysectomy consistent with Sertoli cells being the primary site of action of FSH in the testis (Dorrington & Fritz, 1973). FSH-R mRNA is expressed in Sertoli cells (Heckert & Griswold, 1991; Kliesch *et al.*, 1992). Purified Sertoli cells have been shown to respond to FSH by increasing their production of cAMP by activating adenylate cyclase *in vitro* (Fakunding *et al.*, 1976). This rapid increase in cAMP in turn leads to activation of cAMP-dependent protein kinase and phosphorylation of proteins. Interestingly, FSH binding to spermatogonia has been demonstrated using radiolabelled FSH but the significance of this finding is uncertain (Orth & Christensen, 1978).

FSH has been shown to be essential for Sertoli cell proliferation in the foetal rat (Huhtaniemi *et al.*, 1986). Maximal Sertoli cell proliferation occurs at day 18 of gestation in the rat foetus. When day 18 foetuses were decapitated *in utero* or given antiserum to FSH the number of Sertoli cells preparing to divide on day 19, as measured by incorporation of ^{3}H-thymidine, was decreased compared to age matched controls (Orth, 1984). However, the degree of stimulation by FSH of DNA synthesis and mitosis of Sertoli cells decreases with age in the rat (Griswold *et al.*, 1977). The production of cAMP in response to FSH was studied using Sertoli cells isolated from rats of different ages. A dramatic decrease in the response occurred between 18 and 36 days of age and FSH alone had no effect on Sertoli cells from 60 day old rats in the absence of a phosphodiesterase inhibitor (Steinberger *et al.*, 1978). This decrease in response is not thought to be due to a decrease in FSH receptor numbers since these have been shown to increase with age in the rat testis (Bortolussi *et al.*, 1990). When germ cells begin to proliferate and differentiate in the seminiferous epithelium at the onset of spermatogenesis (at about 20 days of age in the rat) the response of the Sertoli cell to FSH declines. FSH is thought to be the major factor involved in initiating spermatogenesis although the precise mechanisms by which it achieves this are unknown.

2.3.5.2 FSH and the control of spermatogenesis

The role of FSH in the adult testis is still unclear (for review see Sharpe, 1994). Active immunisation with FSH in sexually mature rhesus monkeys was observed to severely impair spermatogenesis resulting in a decrease in testicular volume and tubules containing only Sertoli cells and spermatogonia (Srinath *et al.*, 1983). However, this study was continued for 4.5 years and at the end of this period spermatogenesis had recovered in the treated animals although only qualitatively suggesting other factors were becoming more effective at supporting spermatogenesis. In normal men suppression of FSH by chronic hCG administration was shown to decrease sperm count but did not cause azoospermia, and sperm motility and morphology remained normal throughout the study (Matsumoto *et al.*, 1986). Readministration of FSH restored the sperm count to within the normal range. This study demonstrated that in man FSH is not an absolute requirement for the maintenance of complete spermatogenesis but is necessary for the maintenance of quantitatively normal sperm production. In contrast to these results in the monkey and human, neutralisation of FSH in adult rats appears to have only minor effects on testicular morphology or fertility (Dym *et al.*, 1979).

It has been shown in normal cynomolgus monkeys (*Macaca fascicularis*) that daily administration of FSH increases the proliferation of A_p spermatogonia and in time causes an increase in the numbers of pachytene spermatocytes and round spermatids (van Alphen *et al.*, 1988). No degeneration of germ cells was observed in this study and if left to proceed through enough cycles it is proposed that the FSH treatment will ultimately result in an increase in sperm count. FSH may be having a direct effect on the spermatogonia since it has been proposed that these cells express receptors for FSH (Orth & Christensen, 1978). The number of differentiating spermatogonia in the rat does not appear to be regulated in this way by FSH perhaps providing an explanation for the differences in response to FSH neutralisation in these species (de Rooij *et al.*, 1989). After hypophysectomy in adult rats spermatogenesis was shown to regress but FSH treatment only partially restored spermatogenesis unless given in conjunction with testosterone (Bartlett *et al.*, 1989). These results are in contrast to the neutralisation studies but it is thought that this may be due to residual production of testosterone by the intact Leydig cells in the hypophysectomised animals. The role of FSH in maintaining spermatogenesis in the adult mammal is still a matter for debate but it is probable that regulation is not by one factor alone but involves the interaction of several factors produced either by cells outwith or those within the testis.

2.3.5.3 LH stimulation of steroidogenesis

High affinity binding sites for LH have been found only in the interstitial compartment of the testis (Catt *et al.*, 1972). Seminiferous tubules appear not to be the main site of androgen synthesis since when ^{3}H-cholesterol, thought to be the precursor of testosterone, was injected into the testis all the radioactivity was found in the interstitial cells (Parvinen *et al.*, 1970). Furthermore only interstitial cells carry out the conversion of ^{3}H-7α-cholesterol to ^{3}H-testosterone (Hall *et al.*, 1969; see also Fig.3). Isolated rat Leydig cells have been shown to respond to hCG or LH by increasing cAMP and testosterone biosynthesis (Dorrington & Fritz, 1973; Moyle & Ramachandran, 1973).

This response of Leydig cells to LH stimulation is evident as early as day 15 of gestation in the rat foetus (Gangnerau *et al.*, 1982).

In the rat, testosterone is secreted episodically by Leydig cells. In most cases an increase in testosterone production is preceded by an LH pulse and a rise in testosterone is followed by a trough in pulsatile LH secretion (Ellis & Desjardins, 1982). This pattern of secretion demonstrates LH-stimulated steroidogenesis and the steroid feedback from the testes to the hypothalamic-pituitary axis (see Fig.5). LH binding to Leydig cells increases cAMP levels and this leads to activation of a protein kinase and a cascade of protein phosphorylation (Dufau *et al.*, 1981). It has been proposed that the proteins phosphorylated by this kinase could be necessary for cholesterol transport and side-chain cleavage which causes conversion of cholesterol to pregnenolone (see section 2.2.2.1 and Fig.3). Therefore, stimulation of testosterone synthesis by LH appears to be achieved by the activation of cholesterol side-chain cleavage, the rate limiting step in steroidogenesis (Caron *et al.*, 1975).

2.3.5.4 Testosterone and spermatogenesis

Testosterone treatment alone has been shown to maintain qualitatively normal spermatogenesis in adult hypophysectomised rats (Bartlett *et al.*, 1989) but is less effective in other primates (Marshall *et al.*, 1986). To achieve normal intratesticular levels of testosterone in hypophysectomised, EDS-treated (Leydig cell depleted) rats it is necessary to have supranormal blood levels (Sharpe, 1994). When normal intratesticular levels of testosterone are achieved in treated rats spermatogenesis is efficiently maintained (Sharpe *et al.*, 1988). However physiological blood levels of testosterone are unable to support spermatogenesis in the absence of FSH (Kerr *et al.*, 1992). Testosterone and FSH may therefore act synergistically to support spermatogenesis in the adult animal (Kerr *et al.*, 1992). The ability of FSH to increase Sertoli cell androgen receptors may be one way in which this synergism is achieved (Sanborn *et al.*, 1991).

Androgen receptors have been localised not only in Sertoli cells but are also found on peritubular myoid cells and interstitial cells of the rat and human (Sar *et al.*, 1990). The presence of androgen receptors in germ cells is still being debated with several reports claiming germ cells bind androgen (Sanborn *et al.*, 1975; Wright & Frankel, 1980) while other groups failed to find any expression of the receptor (Grootegoed *et al.*, 1977; Sar *et al.*, 1990). The total number of androgen receptors in the rat testis was shown to increase between days 25 and 60 (Buzek & Sanborn, 1988). This increase was thought to be due to an increase in Leydig cell number and to an increase in receptor concentration on Sertoli cells. This period of development coincides with a decrease in Sertoli cell responsiveness to FSH and it has been postulated that these cells switch from being primarily FSH responsive to androgen responsive (Steinberger *et al.*, 1978; Jégou *et al.*, 1982).

Stages VII-VIII of the spermatogenic cycle are believed to be particularly dependent on stimulation by androgen since germ cells at these stages are the first to degenerate after androgen withdrawal (EDS treatment; Kerr *et al.*, 1993). Furthermore, Leydig cells adjacent to tubules at these stages were reported to be larger than those surrounding other stages of the cycle although this remains to be confirmed (Bergh, 1983). The concentration of androgen receptors in staged microdissected seminiferous tubules from the rat is highest at stages IX-XII (Isomaa *et al.*, 1985) but all receptors in the testis are thought to be occupied by testosterone since the concentration of this androgen in the testis is very high (see Sharpe, 1994 for review; Grino *et al.*, 1990). Whilst it is generally agreed that high levels of androgen are necessary for the support of spermatogenesis other mechanisms may exist by which testosterone acts within the testis but these remain to be elucidated (Sharpe *et al.*, 1993; Sharpe, 1994).

2.4. Paracrine interactions in the testis

There is a growing body of evidence demonstrating the importance of cellular interactions in the regulation of testicular function (Fig.5). Numerous studies carried out both *in vitro* and *in vivo* have demonstrated morphological and functional interactions between Sertoli cells, germ cells, Leydig cells, peritubular myoid cells and macrophages (for review see Sharpe, 1993). Some of the evidence is reviewed in the following sections.

2.4.1 Sertoli cell-germ cell interactions

There is evidence for the importance of Sertoli cells in the regulation of germ cell development as described in section 2.2.1 and also for a role for germ cells in the modulation of Sertoli cell function. The function of the Sertoli cell has been shown to change in a cyclical manner according to the stage of the spermatogenic cycle (see Parvinen, 1993 for review). This cyclicity of function is thought to be a consequence of the changing complement of germ cells at the different stages of the spermatogenic cycle since all stages are thought to be exposed to the same hormonal environment. Evidence for this comes both from *in vitro* and *in vivo* studies and shows both structural and functional interactions between the cell types.

Morphological interactions between Sertoli cells and germ cells. It has been well established that Sertoli cells and germ cells interact both physically and chemically via

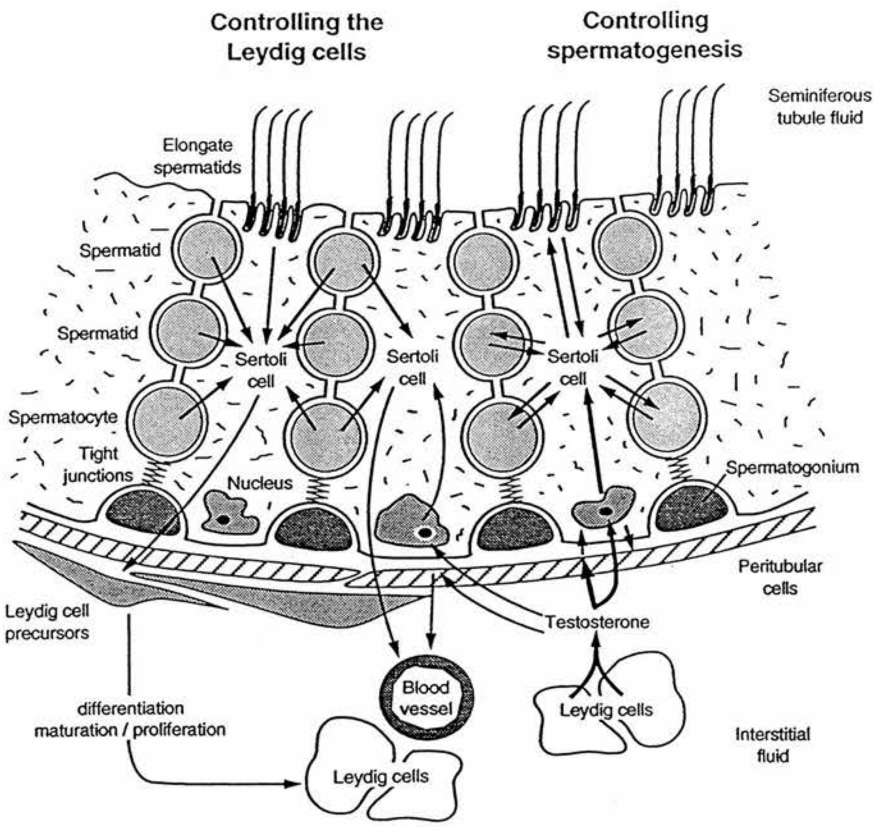


Figure 5. Cell-cell interactions in the testis. Schematic of interactions between cells of the seminiferous tubule and the interstitial space of the testis and how they are involved in the control of Leydig cell function and spermatogenesis. *Adapted from Sharpe, 1993.*

an array of structural devices. Some of these devices are commonly found between cells in other tissues and others are unique to the testis. Desmosome-like junctions are present mainly between pachytene spermatocytes and Sertoli cells and are rarely seen on spermatids (Russell, 1980). Normal desmosomes function to bind cells together and this may be the case in the testis. It has been proposed that they may act to hold the germ cells in correct association with the Sertoli cell as its cytoplasm changes shape to allow movement of the germ cells within the seminiferous epithelium (Russell, 1993a). Gap junctions have also been found between Sertoli cells and germ cells at almost all stages of development (McGinley *et al.*, 1979). These structures are areas where the Sertoli cell and germ cell membranes converge but leave a space which is usually thought to allow passage of small molecules between the two cells (Enders, 1993). It is possible that the presence of gap junctions between Sertoli cells and germ cells allow passage of molecules between the cells which are important for mutual regulation.

The ectoplasmic specialisation is a unique type of junction between Sertoli cells and germ cells (Russell, 1977). All germ cells more mature than, but including, pachytene spermatocytes are found in association with these specialisations (Russell, 1980). However, it appears that it is mainly elongate spermatids which are bound by these as trypsin treatment is required for removal of these cells from the ectoplasmic specialisation (Romrell & Ross, 1979). The function of the ectoplasmic specialisations is uncertain. The spermatids most closely associated with these junctions are found embedded in deep recesses of the Sertoli cell cytoplasm at certain stages of the spermatogenic cycle (Russell, 1993a). The spermatids move within the seminiferous epithelium and it has been postulated that the ectoplasmic specialisation may be involved in this movement. It is also thought that the ectoplasmic specialisation plays a role in sperm release possibly by the loss of specialisations at the head of the elongate spermatid and its movement from the recesses of the Sertoli cell cytoplasm (Russell, 1991). This possibility requires further study to be confirmed.

Finally, tubulobulbar complexes are found between Sertoli cells and germ cells (Russell, 1980). These appear at the same time and in the same regions from where the ectoplasmic specialisations are disappearing (Russell, 1991). They appear in the last few days before sperm release between the mature spermatids and the Sertoli cells and are formed in areas of coated pits on the Sertoli cell plasma membrane with projections of the spermatids elongating to fill these invaginations. These complexes may be involved in the elimination of residual cytoplasm from the spermatid prior to its release as these are sites of active Sertoli cell phagocytosis (see section 2.2.1.4). The tubulobulbar complexes begin to disappear just prior to sperm release but would appear to anchor the spermatids in the Sertoli cells to prevent premature release.

Evidence for functional interactions; in vitro studies. There have been numerous studies on germ cell-Sertoli cell interactions carried out using *in vitro* systems. Some of these are reviewed in this section.

Secretion of transferrin by the Sertoli cell has been shown to be influenced by the presence of germ cells (see chapter 5 for further discussion). Removal of germ cells from cultures of Sertoli cells from 20 day old rats caused a significant inhibition of FSH stimulated transferrin secretion compared to cultures still contaminated with germ cells (Le Magueresse *et al.*, 1988). Addition of enriched fractions of spermatocytes or spermatids, or media from cultures of these cells, to Sertoli cell only cultures stimulated transferrin production. Co-culture with liver epithelial cells also stimulated Sertoli cell transferrin secretion but this effect was not observed using conditioned medium from cultures of these cells. These data are consistent with a factor/s produced specifically by germ cells stimulating transferrin secretion but since this stimulation was greater in co-culture than with conditioned media alone the effect may be mediated by cell contact as well as by a secreted factor/s. Secretion of transferrin by Sertoli cells from 18 day old rats cultured in a bicameral chamber system was increased by addition of proteins secreted by pachytene spermatocytes (PSP) and round spermatids (RSP) (Djakiew & Dym, 1988; Onoda & Djakiew, 1990). Interestingly, RSP stimulated total Sertoli cell protein secretion predominantly in an apical direction which would correspond to the localisation of the germ cells in an *in vivo* situation. The regulation of transferrin production probably occurs at least in part at the level of gene transcription as transferrin mRNA levels in cultured Sertoli cells are stimulated by incubation with a crude fraction of germ cells (Stallard & Griswold, 1990).

Addition of fractions enriched in pachytene spermatocytes, early spermatids or cytoplasts from elongated spermatids was also shown to stimulate ABP secretion by Sertoli cells in culture (Le Magueresse & Jégou, 1988a). This stimulation was greatest with pachytene spermatocytes and is thought to involve a germ cell protein/s since it could be abolished by heat or trypsin treatment of pachytene spermatocyte or round spermatid conditioned media. In contrast to these results another study has shown that cell contact is necessary for stimulation of ABP secretion by Sertoli cells from 25 day old rats (Galdieri *et al.*, 1984). Prevention of contact between Sertoli cells and pachytene spermatocytes using a layer of agar over the Sertoli cell monolayer inhibited this increase in ABP secretion. Round spermatids did not affect secretion and this may have been due to the failure of these cells to adhere to or recognise Sertoli cells from immature animals (Ziparo *et al.*, 1980). The inconsistency of these results may be due to differences in the culture systems.

Inhibin has also been shown to be under the control of germ cells *in vitro.* Addition of a crude fraction of germ cells isolated from adult rats stimulated the secretion of inhibin from immature Sertoli cells in culture as measured by radioimmunoassay specific for α-inhibin (Pineau *et al.*, 1990). This stimulation appeared to be due to round spermatids as conditioned media from these cells reproducibly increased inhibin production while pachytene spermatocyte conditioned media had less effect. The expression of α-inhibin mRNA in Sertoli cells was also increased by addition of round spermatid conditioned media.

The direction of secretion of Sertoli cell proteins can be influenced by the presence of germ cells. Secretion of SGP-2, α_2-macroglobulin (a protease inhibitor) and testins (Sertoli cell proteins the secretion of which is stimulated by testosterone) from 20 day old Sertoli cells cultured in a bicameral chamber was measured by Grima and co-workers (1992). SGP-2 was secreted predominantly in an apical direction while α_2-macroglobulin and testins were secreted both apically and basally. Addition of germ cell conditioned media to the apical chamber of the culture system caused an inhibition of apical secretion of SGP-2 and testins and a small increase in their basal secretion. Germ cells have been reported to have no effect on the level of SGP-2 mRNA in Sertoli cells indicating that regulation may occur at the level of translation (Stallard & Griswold, 1990). The secretion of α_2-macroglobulin was stimulated in both directions. The significance of this differential regulation of the direction and level of secretion of Sertoli cell proteins by germ cell conditioned medium is unknown.

The studies reviewed in this section all involved the use of Sertoli cells isolated from approximately 20 day old rats in co-culture with germ cells isolated from adult animals. At 20 days the most mature germ cell type present in the rat testis are early pachytene spermatocytes (Russell *et al.*, 1987) and this makes it easier to isolate a pure fraction of Sertoli cells. However, the *in vitro* effects of germ cells on Sertoli cell secretory activity is influenced by the age of rat from which the Sertoli cells were isolated. In cells isolated from animals at 18- or 26-days of age hypotonic treatment to remove contaminating germ cells caused a decrease in ABP and transferrin secretion compared to untreated controls while hypotonic treatment of Sertoli cells from 10 day old rats had no effect on secretion of these proteins (Castellón *et al.*, 1989). There is also an age related increase in secretion of these proteins but this was less pronounced when germ cells were removed from the cultures. Sertoli cells from 10 day old rats were found to bind pachytene spermatocytes to a lower extent than Sertoli cells from the other two age groups. This may be due to the fact that *in vivo* these Sertoli cells would not have been exposed to germ cell types as advanced as this and have not yet developed the appropriate recognition and adhesion molecules. The interpretation of the studies reviewed above and their significance to actual interactions occurring in the adult *in vivo* must therefore be undertaken with this limitation in mind.

Evidence for functional interactions; in vivo studies. Study of germ cell modulation of Sertoli cell function *in vivo* has been approached using several different experimental systems. These have included treatment with testicular toxicants such as methoxyacetic acid (MAA) which destroys pachytene spermatocytes (Bartlett *et al.*, 1988), γ-irradiation of the testis to destroy mainly spermatogonia (Pinon-Lataillade *et al.*, 1988), hypophysectomy followed by hormonal treatments (Roberts *et al.*, 1991) and local heating of the testis (Jégou *et al.*, 1984). These treatments all have the advantage over *in vitro* studies in that they can be carried out in adult animals and they usually disrupt only one of the many cellular interactions occurring in the testis.

Low dose γ-irradiation of the testis primarily causes damage to type A spermatogonia but this in turn causes a progressive reduction in the number of later germ cells through a maturation-depletion process (Pinon-Lataillade *et al.*, 1988). There is no obvious direct effect on the Sertoli cells. At selected times after irradiation different germ cell types will be absent from the seminiferous tubules and the effect of their absence can be studied. ABP secretion into the epididymis was decreased approximately 70 days after irradiation. This decrease coincided with the depletion of late spermatids from the seminiferous epithelium. The serum levels of FSH also increased at this time and this has been proposed to be due to an effect of late spermatids on the secretion of inhibin by the Sertoli cells. Evidence in support of this hypothesis has been provided in a study using the testicular toxicant MAA to deplete germ cells from the seminiferous tubules (Allenby *et al.*, 1991). Levels of FSH in the blood were increased 21-35 days after MAA treatment at a time when late spermatids were depleted from the tubules. Plasma levels of immunoreactive inhibin were significantly decreased at these time points which would account for the increase in FSH levels. Using this experimental model ABP has been shown to increase in the absence of late spermatids, as shown in the irradiation studies, but also when pachytene spermatocytes were depleted from the tubules (Bartlett *et al.*, 1988). However, whilst round spermatids did not influence inhibin secretion from adult Sertoli cells, they did cause an increase in the levels of α-inhibin mRNA *in vitro* in Sertoli cells isolated from 20-day old rats (Pineau *et al.*, 1990). These data support the argument that the response of the Sertoli cell to germ cells is dependent on the stage of maturation of the Sertoli cells and that the modulation of Sertoli cell function is transferred to the most advanced generation of germ cells present within the seminiferous epithelium (Jégou, 1991).

Hypophysectomy of adult rats causes a decline in intratesticular testosterone levels and a reduction in the number of germ cells in the testis. Testosterone levels can be restored to normal by implantation of testosterone containing capsules and in time the germ cells will repopulate the testis. This model has also been used to study the influence of germ cells on specific Sertoli cell functions (Roberts *et al.*, 1991). It was shown that transferrin mRNA levels were decreased 4 weeks after hypophysectomy while those for SGP-2 were unaffected. Administration of testosterone did not restore transferrin mRNA expression although after 4 weeks most pachytene spermatocytes had returned and 8 weeks after treatment the majority of tubules also had a full complement of round spermatids. At both these time points transferrin mRNA levels were increased compared to those in the hypophysectomised, untreated rats. Again SGP-2 mRNA levels were unaffected showing that this is not a general influence on Sertoli cell secretion. The effect of testosterone withdrawal and germ cell depletion on mRNAs for these proteins has also been described (Roberts *et al.*, 1992). This was achieved by administration of testosterone and oestradiol filled capsules to intact rats to inhibit LH synthesis by the pituitary and to decrease intratesticular testosterone production. This experimental model avoids the complications caused by removal of the pituitary gland which may secrete factors other than the gonadotrophins which could be involved in regulating Sertoli cell function. The results obtained from this study confirmed those from the previous study using hypophysectomised rats as transferrin mRNA levels were not influenced by testosterone concentration but were dependent on the presence of germ cells in the testis.

Sertoli cell production of testins is also controlled by germ cells *in vivo* (Jégou *et al.*, 1993). Using γ-irradiation of adult rats the levels of testin secretion is increased at all time points when germ cells are missing. The increase was greatest when elongate spermatids were depleted but there were significant increases in the absence of spermatogonia, spermatocytes and early spermatids suggesting that testins production by Sertoli cells is normally regulated negatively by germ cells.

Elongate spermatids have also been reported to play a role in regulating the production of seminiferous tubule fluid by the Sertoli cell which is important for the control of protein secretion from the Sertoli cell and also for sperm transport from the testis (section 2.2.1.4; Jégou *et al.*, 1984). Overall protein secretion from isolated stage VI-VIII seminiferous tubules was reduced when pachytene spermatocytes, round spermatids or elongate spermatids were depleted by treatment with MAA (McKinnell & Sharpe, 1992). In this study two dimensional polyacrylamide gel electrophoresis analysis demonstrated that selected proteins were specifically and differentially affected by the absence of the different germ cell types. Therefore, this study provides further evidence for the importance of cellular interactions in testicular functions.

Mechanism of germ cell influence on Sertoli cell function. The mechanisms by which germ cells influence Sertoli cell function are still poorly understood but there are several potential forms of interaction.

1) **Germ cells may secrete factors** which modulate Sertoli cell function and evidence for this has been provided in the preceding sections.

(a) Transferrin mRNA in Sertoli cell cultures was stimulated by germ cell conditioned medium (Stallard & Griswold, 1990). This stimulatory activity was partially isolated and was found to be due to protein/s in the 10-30kDa molecular weight fraction. Recently this protein/s has been further characterised and tentatively identified as basic fibroblast growth factor (bFGF) in germ cell conditioned medium (Suk Han *et al.*, 1993). This growth factor was shown to stimulate transferrin secretion from immature Sertoli cells. It was localised in spermatogonia, pachytene spermatocytes, Leydig cells and Sertoli cells by immunostaining. The bFGF receptor was expressed in a stage specific manner on Sertoli cells but was also found on late spermatids and Leydig cells. This factor is therefore proposed to be important, not only for germ cell-Sertoli cell communication but also for interactions between several other cell types in the testis.

(b) Nerve growth factor mRNA is expressed in spermatocytes and its protein product is found in these and more mature germ cell types (Persson *et al.*, 1990). Its receptor is found on Sertoli cells and is expressed specifically at stages VI-VIII of the spermatogenic cycle. Nerve growth factor could therefore be another germ cell product involved in the modulation of Sertoli cell function.

(c) Numerous other studies have identified stimulatory or inhibitory activity in germ cell conditioned media for Sertoli cell function but the proteins remain to be characterised.

2) **Contact between Sertoli cells and germ cells** may be important in their interaction. Late spermatids have been shown to influence Sertoli cell function in several ways but it is unlikely that new gene expression is involved as these cells are transcriptionally inactive (Hecht, 1990). Antibodies directed against a liver plasma membrane protein were found to cross react with epitopes found on Sertoli cells and spermatocytes (Corlu *et al.*, 1992). Stimulation of transferrin secretion by addition of pachytene spermatocytes to Sertoli cells in culture was inhibited by the addition of antibodies directed against this protein. This study provides evidence for the importance of cell-cell contact in regulation of Sertoli cell function.

3) **A direct cytoskeletal link between spermatid-Sertoli cell contacts and the nuclear transcription machinery** has been proposed (Jégou *et al.*, 1992). It has been shown that marked changes in the shape of the Sertoli cell occur partly due to the movement of germ cells within its cytoplasm during spermatogenesis (Russell, 1993c). Changes in the shape of cells could be translated via the cytoskeleton to the nucleus leading to a physical modulation of gene transcription. However, there is no direct evidence for a contribution of this model to germ cell modulation of Sertoli cell gene expression.

4) **A role for residual bodies and tubulobulbar complexes** in the regulation of Sertoli cell function has been proposed recently (Jégou *et al.*, 1992). Phagocytosis of residual bodies by Sertoli cells in the rat occurs at the same time as other important events in spermatogenesis such as mitosis of A type spermatogonia and the entry of spermatocytes into meiotic prophase. Residual bodies are known to contain RNA which could be delivered to the Sertoli cell for translation but this remains speculation (Jégou, 1991). It has also been shown that phagocytosis of residual bodies by immature Sertoli cells in culture causes release of interleukin-1 by the Sertoli cell (Gérard *et al.*, 1992). IL-1 is produced by seminiferous tubules from adult rats and its production is stage specific being highest at stages VII and VIII which correlates with the stages of residual body formation (Syed *et al.*, 1988). IL-1 can stimulate DNA synthesis in seminiferous tubules (Parvinen *et al.*, 1991) and as a cytokine could possibly have numerous other biological effects on germ cells.

2.4.2 Sertoli cell-Leydig cell interactions

The most obvious and well studied form of interaction between Leydig cells and Sertoli cells is via the steroid hormone testosterone (see section 2.3.5.3). Leydig cells also express POMC mRNA and the peptides derived from this have been shown to influence Sertoli cell function (see section 2.2.2.1).

Whilst the production of factors by the Leydig cell have been shown to influence Sertoli cell function, several studies have also pointed to a role for the seminiferous tubules in the regulation of Leydig cell function. Administration of the antifertility drug cyproterone acetate causes regression of the seminiferous tubules. This was shown to be accompanied by Leydig cell hyperplasia and an increase in Leydig cell smooth endoplasmic reticulum (SER) which determines the steroidogenic capacity of the cell (Aoki & Fawcett, 1978). This influence on Leydig cells was not due to an increase in gonadotrophin levels as it has also been shown that in unilaterally cryptorchid animals hypertrophy was only observed in Leydig cells in the abdominal testis although both testes are presumed to be exposed to the same gonadotrophin levels (Risbridger *et al.*, 1981). Stimulation of the damaged testis with hCG *in vitro* showed an increased responsiveness as judged by testosterone synthesis which had been previously proposed due to the increase in SER volume.

Other studies which have suggested a role for seminiferous tubules in modulating Leydig cell function have pointed to the existence of a cycle in the size of Leydig cells as evidence. In the rat adult Leydig cells adjacent to tubules at stages VII-VIII were found to be much larger than those next to tubules at other stages (Bergh, 1983). This difference in size was abolished in cryptorchid testis and was not apparent in 43 day old rats but was noticeable by 47 days of age (Bergh, 1985). This development of a Leydig cell 'cycle' could be due to maturation of the Sertoli cell or the appearance of step 19 spermatids in the seminiferous epithelium as these are not present in 43 day old rats. However, a later study did not show any difference in Leydig cell size in the rat or monkey although an increase in smooth endoplasmic reticulum volume was demonstrated in Leydig cells adjacent to stage VII tubules (Fouquet, 1987).

FSH, acting via the Sertoli cells, has been shown to stimulate LH receptor number and the capacity for hCG stimulated testosterone secretion by Leydig cells from immature hypophysectomised rats *in vitro* (Kerr & Sharpe, 1985). Sertoli cells or Sertoli cell conditioned medium from the immature rat also stimulated Leydig cell steroidogenesis *in vitro* (Papadopoulos *et al.*, 1987). An influence of whole isolated seminiferous tubules on Leydig cell testosterone production *in vitro* has also been shown by several studies but the results are less clear for although tubules at all stages of the cycle were shown to decrease testosterone synthesis in a crude population of Leydig cells the same study showed stimulation of testosterone production when a Percoll-purified population of Leydig cells was used (Parvinen *et al.*, 1984). Co-perifusion of Leydig cells and seminiferous tubules was shown to increase testosterone production whereas seminiferous tubules had no effect on testosterone synthesis when co-incubated with Leydig cells (Bartlett *et al.*, 1987). These conflicting results could be due to an influence of the cell preparation and incubation procedures or could point to the presence of more than one seminiferous tubule factor involved in the regulation of Leydig cell steroidogenesis.

There are several candidates for the Sertoli cell factors involved in regulation of Leydig cell function. GnRH receptors have been identified in interstitial tissue and are thought to be present specifically on the surface of Leydig cells (Bourne *et al.*, 1980). Addition of GnRH and GnRH agonists to Leydig cells causes an initial, brief stimulation of steroidogenesis followed by an inhibition of testosterone synthesis (Sharpe *et al.*, 1983). It has been proposed that a GnRH-like factor is produced by Sertoli cells in the rat and macaque testis and may be involved in the regulation of Leydig cell function (Sharpe *et al.*, 1981). Human Sertoli cell conditioned medium contains a factor which has been shown to stimulate Leydig cell steroidogenesis (Papadopoulos, 1991). This protein has been isolated and has a molecular weight of approximately 80kDa but has not been further characterised.

Production of a Sertoli cell factor/s influencing Leydig cell function may also be under the control of germ cells. Secreted proteins from immature Sertoli cells stimulated steroidogenesis in purified rat Leydig cells and in MA-10 mouse tumour

(Verhoeven & Cailleau, 1988). The inhibition of aromatase activity and stimulation of ABP was not greatly affected by peritubular cells alone but on addition of androgens the influence of peritubular cells was significantly increased. These results could be obtained using conditioned media from peritubular cells in place of co-culture. It is possible that the factors responsible for ABP stimulation and aromatase inhibition in Sertoli cells are identical and may be P-Mod-S. These results demonstrate an interaction between Leydig cells, Sertoli cells and peritubular cells in the testis. Androgen produced by the Leydig cells may act directly on the Sertoli cell to regulate its function but is also shown to act on peritubular cells which in turn produce a factor/s which influences Sertoli cell function.

Transforming growth factor-α mRNA is expressed both in Sertoli cells and peritubular cells (Skinner *et al.*, 1989b). This growth factor binds to EGF receptors with high affinity and these receptors have been found only on peritubular cells in the testis. TGFα stimulated peritubular cell proliferation in culture but had no effect on Sertoli cells. Transferrin secretion by immature Sertoli cells in culture was not stimulated by TGFα but in co-culture with peritubular cells this growth factor did stimulate transferrin production. TGFß is also found in Sertoli cells and peritubular cells (Skinner & Moses, 1989). This factor was not seen to influence Sertoli cell function *in vitro*. However, it did cause an increase in peritubular cell protein secretion and stimulated peritubular cell migration and formation of cell clusters in Sertoli cell-peritubular cell co-cultures. This may have been due to an effect on secretion of extracellular matrix proteins by peritubular cells. These results provide further evidence for potential paracrine interactions between Sertoli cells and peritubular cells in the testis.

2.5. Aims of this thesis

To date there has been an enormous amount of data generated in an attempt to understand the normal process of spermatogenesis. Some of that data has been reviewed in this chapter. Control of testicular function by factors of extratesticular origin such as FSH and LH has long been studied and their importance has been established. In contrast, study of cell interactions in the testis has developed relatively recently. In spite of this there have been numerous studies undertaken which have suggested that the different cell types in the testis interact to control their development and specific functions. As has been stated in the preceding sections the majority of these studies have been carried out *in vitro* using isolated testicular cells in culture and they have provided strong evidence for the importance of cellular interactions in the maintenance of the normal process of spermatogenesis. However, the use of an *in vitro* system provides an entirely artificial environment for the testicular cells and interpretation of the results obtained is complicated. An experimental model which maintains the majority of normal cellular associations in the testis is required for a more accurate study of the normal physiological situation. The model adopted in the present study was the depletion of specific germ cell types from the adult rat testis by the use of the testicular toxicant, methoxyacetic acid (MAA). This enabled the investigation of the effect of depletion of a particular germ cell type on specific aspects of Sertoli cell function while all other normal cellular interactions in the testis were maintained. The following chapters of this thesis present the results from such a study.

3. General Materials and Methods

The techniques outlined in this chapter are common to a number of studies in this thesis. Methods specific to individual experiments are described in the relevant chapters.

3.1. Chemicals and suppliers

Molecular biology grade chemicals were obtained from Sigma, Poole, England and IBI, Cambridge, England. All radiolabelled nucleotides were obtained from Amersham, Lewes, England. Enzymes were purchased from Boehringer Mannheim, Buckinghamshire, England or Promega, Southampton, UK. Phenol/chloroform was bought from CAMLAB, Cambridge, England and was pre-buffered with Tris, pH8.0. Autoradiography products were obtained from Kodak supplied by IBI.

3.2. Animals and treatment

3.2.1. Animals

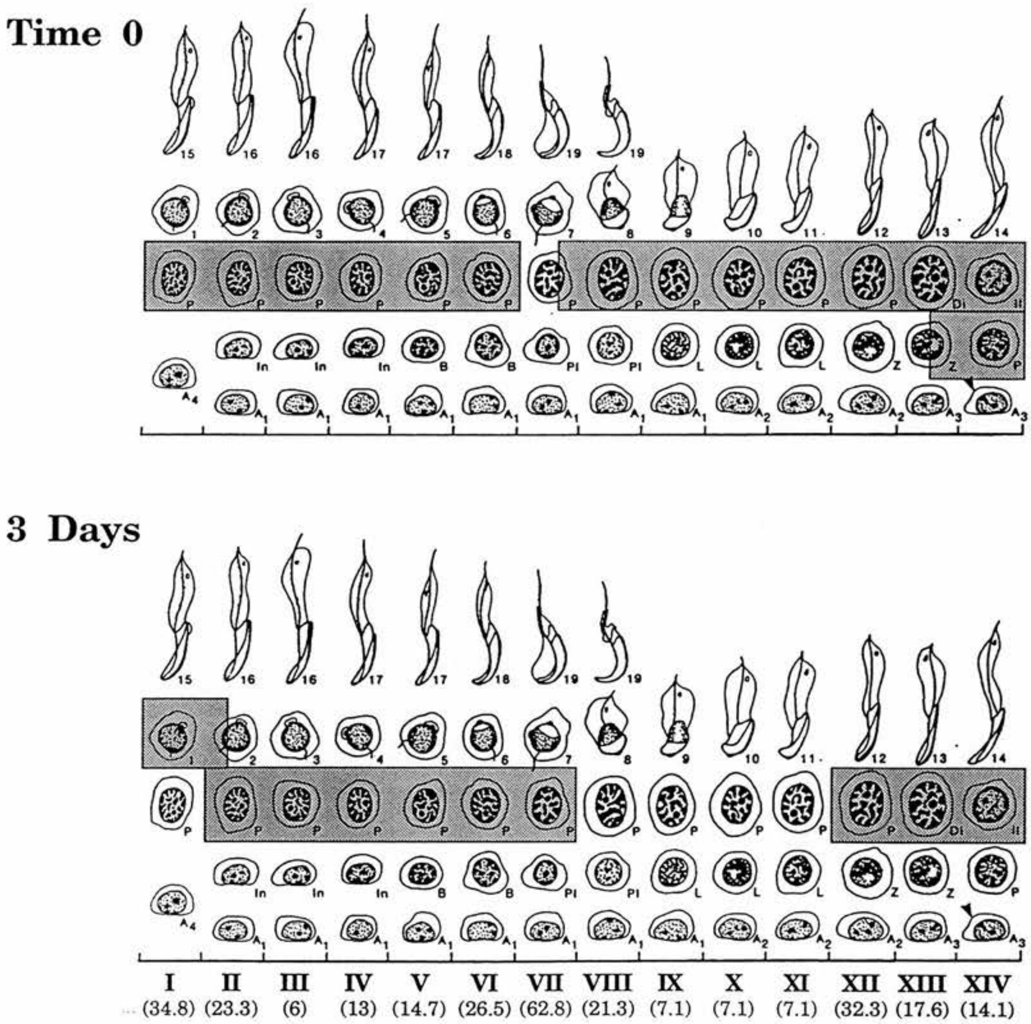
Animals used for these studies were mainly male Wistar rats (adult, aged 65-90 days or immature, aged 16-44 days) bred in the MRC Reproductive Biology Unit in Edinburgh. Adult female Wistar rats were used only for RNA extraction from ovaries. Sertoli cell only rat testis were obtained serendipitously fron an apparently normal, control rat. Rats were maintained in standard conditions of a 12h light : 12h dark cycle and an ambient temperature of 21°C. Food and water were available *ad libitim*. Animals were killed by asphyxiation with CO_2 followed by cervical dislocation.

3.2.2. Treatment

Methoxyacetic acid (MAA; Aldrich Chemical Co. Ltd., Dorset, England) was freshly prepared before administration as follows: 10.8 mls of MAA (pH0.5) were adjusted to pH7.0 - 7.4 with concentrated sodium hydroxide and the volume made up to 45 mls by the addition of normal saline (0.9% w/v sodium chloride). Rats were administered MAA by oral gavage as a single dose of 650 mg/kg. Control animals were given an equivalent volume of saline.

Administration of MAA at this dose level has been shown to result in the selective depletion of 80 - 100% of pachytene and later spermatocytes at all stages of the spermatogenic cycle except early to mid stage VII (Bartlett *et al.*, 1988; Fig.1). Spermatogenesis then proceeds with normal kinetics such that at selected time points after MAA treatment round and then elongate spermatids are selectively absent from the testis due to maturation depletion (Bartlett *et al.*, 1988; Allenby, 1990).

At certain time points after MAA treatment, specifically 3, 7, 14, 21, 28 and 42 days, animals were sacrificed and testes removed for RNA extraction (section 3.3) or fixed for *in situ* hybridisation (sections 3.9 and 3.10).



Stages of the spermatogenic cycle
(Duration of each stage , in hours, shown in brackets)

Figure 1. Schematic representation of germ cell depletion on MAA treatment. Diagram of effect of oral administration of 650mg/kg methoxyacetic acid to adult male rats. Hatched areas show germ cells depleted at various time points after treatment with MAA as determined by Bartlett *et al.*, 1988. *Adapted from Allenby, 1990.*

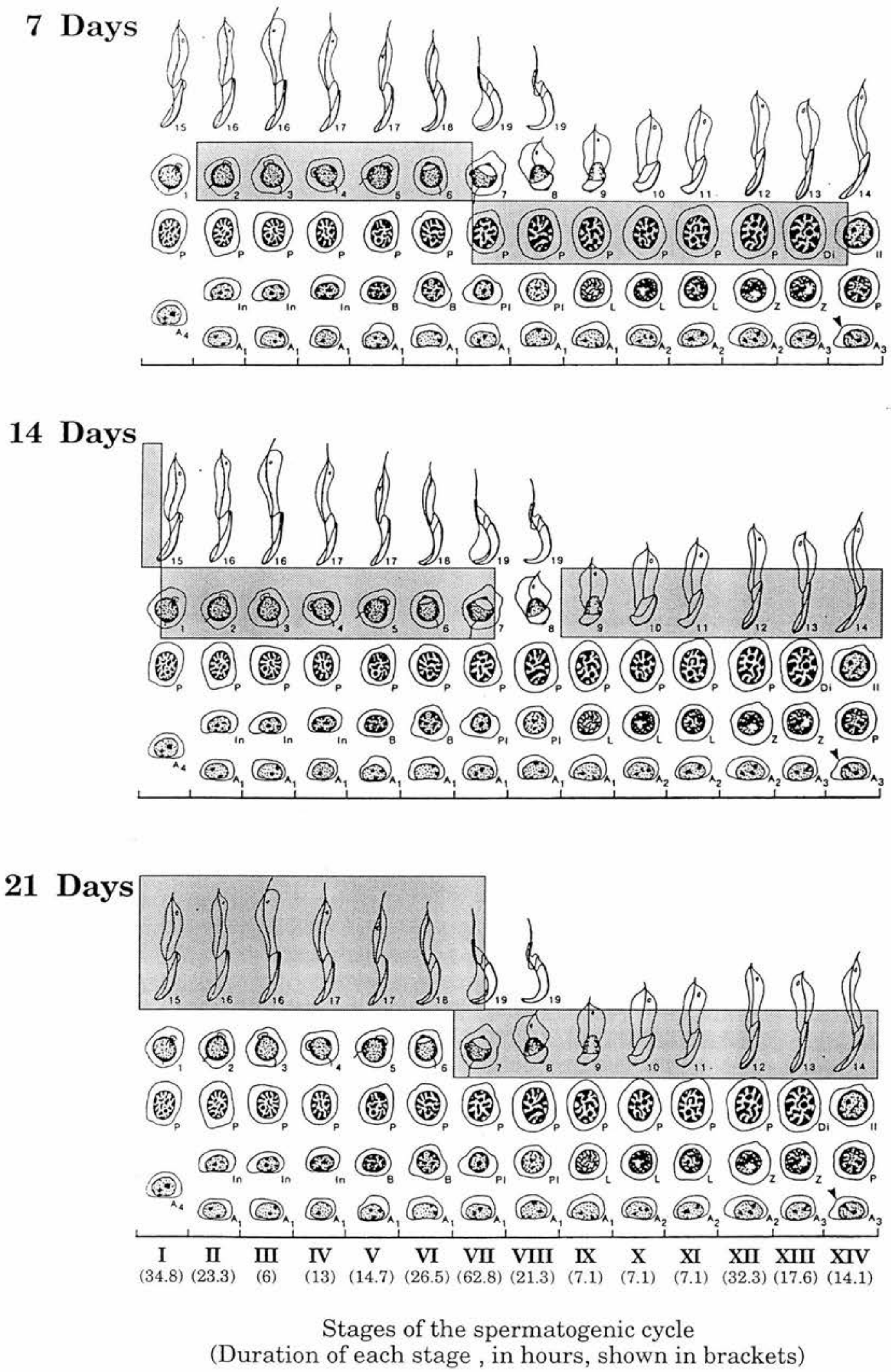
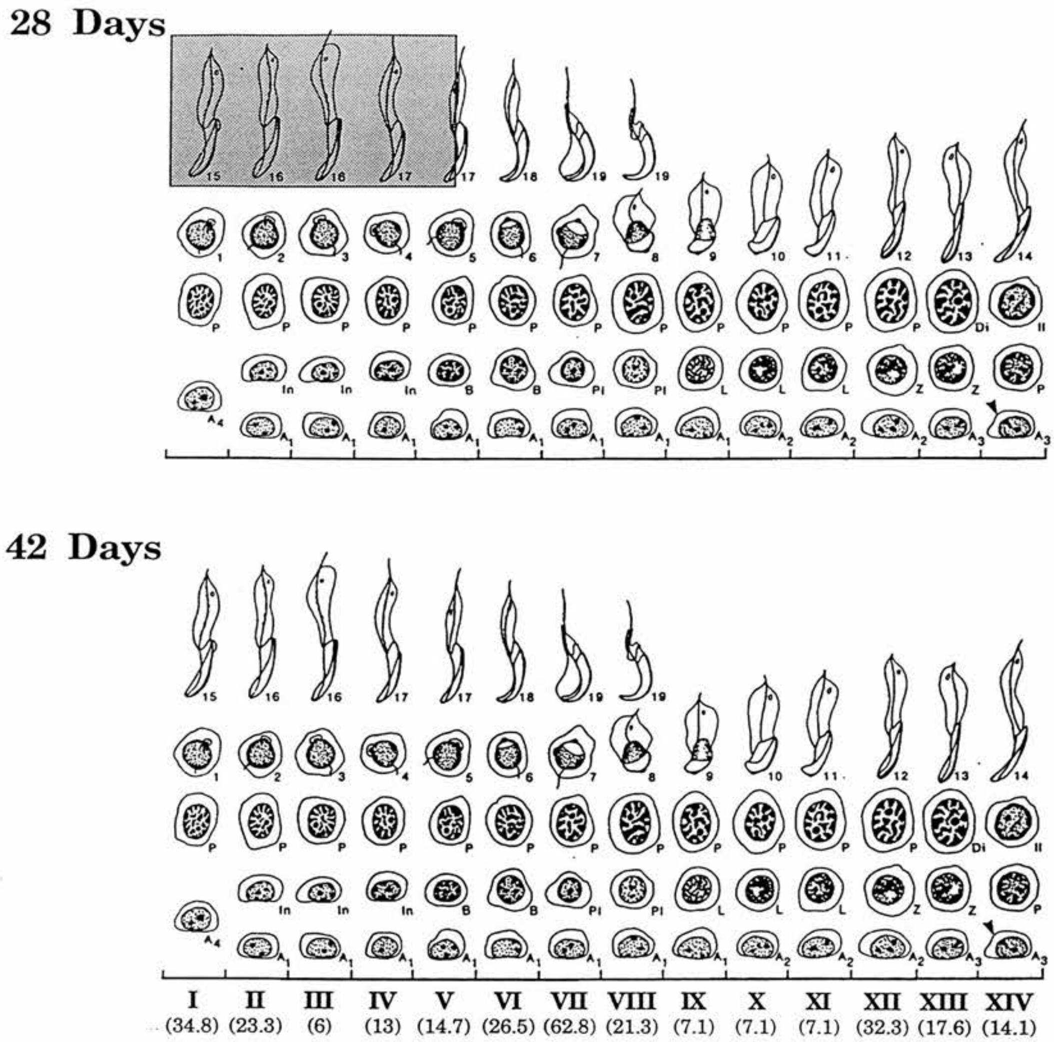


Figure 1 cont. Schematic representation of germ cell depletion on MAA treatment.



Stages of the spermatogenic cycle
(Duration of each stage , in hours, shown in brackets)

Figure 1 cont. Schematic representation of germ cell depletion on MAA treatment.

3.3. RNA extraction and separation

3.3.1. Dissection of tissue

Testis were removed from asphyxiated rats by dissection and were decapsulated. The testicular vein was removed and the tissue was roughly chopped up before placing each testis in 20ml freshly prepared solution D on ice. Immature rat testis and adult rat ovaries were placed in 5ml solution D while 10ml was used for RNA extraction from epididymal, kidney and spleen tissue. Solution D contained 4M guanidinium thiocyanate, 0.1M ß-mercaptoethanol, 25mM sodium citrate and 0.5% sarcosyl.

3.3.2. Extraction of RNA from fresh tissue

RNA was extracted according to the method of Chomczynski & Sacchi (1987) with appropriate adjustment of volumes. The tissue in solution D was homogenised for approximately 30s until completely dissociated. To this homogenate was added 2ml 2M sodium acetate pH4.0, 20ml water saturated phenol and 4ml chloroform/isoamyl alcohol (49:1). The solution was mixed after each addition and an emulsion was seen to form after the final addition. The mixture was placed on ice for 15min and then centrifuged at 10,000g for 20min at 4°C. The top, aqueous phase containing the RNA was removed to a new tube and an equal volume of cold (-20°C) isopropanol was added. The RNA was precipitated at -20°C for at least 1h, centrifuged at 10,000g for 20min at 4°C, the supernatant was discarded and the RNA pellet redissolved in approximately 1ml solution D. An equal volume of isopropanol was added and the RNA reprecipitated at -20°C for at least 1h. RNA was pelleted by centrifugation (10,000g for 20min), washed in 1ml 75% ethanol, recentrifuged and air dried. The RNA pellet was dissolved in RNase-free water by warming to 65°C for 10min and stored at -70°C.

RNA was scanned at 260 and 280nm on a spectrophotometer. The 260:280 ratio for each sample was calculated to give an estimation of the purity of the RNA. A ratio of 2.0 was taken to be pure. The concentration of the RNA was calculated from the 260nm value where an optical density of 1.0 is equal to 40µg/ml RNA.

3.3.3. Separation of RNA on denaturing agarose gels

RNA was separated on a 1.5% denaturing agarose gel. The gel was prepared by melting 2.25g Seakem agarose (FMC supplied by Flowgen, Sittingbourne, England) in 127ml pure water. This was cooled to about 60°C and 15ml of 10 x running buffer (containing 200mM MOPS, 10mM EDTA and 50mM sodium acetate at pH7.0) plus 8.1ml 37% formaldehyde added. The solution was mixed gently and poured into a gel tray (15 x 20cm) containing a 15 well comb in a fume hood. After setting, the comb was removed and the gel submerged in 1 x running buffer in a Sub-Cell electrophoresis cell (Bio-Rad, Hertfordshire, England).

RNA (15μg) in a volume of no greater than 5μl was prepared by adding 15.6μl of sample buffer and heating at 60°C for 5min. Sample buffer contained 100μl 10 x running buffer, 500μl deionised formamide and 178μl formaldehyde. After heating, 8μl of dye solution containing 7.5% w/v ficoll 400, 0.1% w/v bromophenol blue and 1μl of 1mg/ml ethidium bromide was added to each sample. RNA was loaded into individual wells of the gel and separated by running overnight at 35V or for 5h at 120V.

3.4. Tissue fixation and processing

3.4.1. Fixation

Fixation of the tissue was kindly carried out by Dr. Richard Sharpe as follows.

Animals were perfusion fixed with Bouins' fluid via the dorsal aorta. Bouins fluid was prepared with 500ml 40% formaldehyde, 100ml acetic acid and 2 litres saturated picric acid, and filtered before use. The rats were anaesthetised with diethyl ether (May and Baker, Dagenham, England) and the abdominal cavity exposed. A catheter was inserted into the dorsal aorta and normal saline passed through until the testes blood vessels were seen to clear (approximately 3min). Bouins' fluid was then passed through the vessels for a period of 45min. Testes were removed, decapsulated and weighed. The tissue was cut into slices, immersion fixed in Bouins' fluid for 5 hours and transferred to 70% ethanol for storage before processing for *in situ* hybridisation.

3.4.2. Processing and sectioning of tissue

Tissue was processed through a graded series of alcohols in an automatic 2LE Processor (Shandon Scientific Limited, Cheshire, England) using a standard 20 hour cycle and embedded in paraffin wax. Tissue processing was kindly performed by Mr. Mike Millar.

Glass microscope slides to be used for *in situ* hybridisation were washed, dried and baked at 300°C for 8h. Slides were washed in acetone (BDH Ltd., Poole, England) followed by a 0.25% solution of 3-aminopropyl triethoxysilane (TESPA, Sigma) in acetone, rinsed in filtered distilled water and dried.

Paraffin wax embedded tissue was sectioned to a thickness of 5μm using a hand operated "820" Spencer Microtome (American Optical Corporation) and a D-profile knife. Sections were floated on RNase-free water, transferred onto the treated slides and dried overnight before use. Sections for use in immunostaining were sectioned as above but were floated on distilled water and dried onto untreated slides.

3.4.3. Staining of sections

When tissue was sectioned for *in situ* hybridisation alternate sections from each paraffin block were taken and stained using standard methods (Bancroft & Stevens, 1982) to allow identification of cells and determination of the stages of the spermatogenic cycle. Briefly, sections were cleared in histoclear (National Diagnostics, Manville, New Jersey, U.S.A.) and rehydrated in a decreasing series of alcohols. Sections were treated with 1% periodic acid for 5min and washed well in water. Schiff's reagent was then added to the sections for 7min followed by a further 7min wash with running water. Sections were further stained with haematoxylin, differentiated in acid-alcohol and blued in Scott's tap water. Finally, sections were dehydrated, cleared in xylene and mounted. Staging of tubules on the basis of this staining is described in section 3.13.

3.5 Plasmid preparation and analysis

3.5.1. Growth of bacterial cultures

Plasmids containing cDNA inserts were either obtained from colleagues or were prepared by PCR from testicular cDNA pools and subcloned into a suitable vector. The methods are described in the relevant chapters. Bacteria containing plasmids were cultured in Luria Bertani (LB) broth (appendix I) containing 50μg/ml ampicillin. The broth was inoculated with a bacterial colony from an agar plate or from a glycerol stock and incubated overnight at 37°C shaking at 225revs.

3.5.2. Plasmid preparation from bacterial cultures

Plasmid DNA was isolated from 3ml bacterial cultures by the alkaline lysis method using the 'Magic Minipreps' DNA purification system from Promega.

Briefly, suspensions were centrifuged at 1600g and the supernatant discarded, cells resuspended in buffer containing 50mM Tris/HCl, pH7.5, 10mM EDTA and 100μg/ml RNase and lysed with an equal volume of 0.2M NaOH and 1% SDS. The suspension was neutralised with 2.55M potassium acetate and centrifuged for 5min at 12,000g resulting in sedimentation of bacterial genomic DNA. The supernatant (approximately 600μl) was removed, mixed with 1ml DNA purification resin and passed down a miniprep column which retarded only the plasmid DNA. The column was washed with an ethanol based solution, 50μl TE buffer added and incubated at room temperature for 1min. DNA was eluted from the column by centrifugation at 12,000g for 20s.

3.5.3. Analysis of plasmid DNA quality

The approximate concentration of plasmid DNA was determined by comparison with standards containing a known amount of lambda DNA (range of 0.5ng/µl - 10ng/µl). Standards and samples (diluted 1:10 with pure water) were mixed with an equal volume (5µl) of ethidium bromide and viewed under ultraviolet light.

Purity of plasmid DNA was determined by analysis on a 0.8% agarose minigel (Sambrook *et al.*, 1989). This was prepared using Seakem agarose dissolved in 1x TBE buffer (appendix I). The agarose was melted and approximately 200µg/ml ethidium bromide was added for visualisation of DNA. The gel was poured into a 7cm by 10cm gel tray containing an 8 well comb and submerged in 1 x TBE buffer in 'Minnie the Gel-Cicle' submarine cell (Hoefer, Newcastle, England). Plasmid DNA (1µl) was run in a sample solution containing 1µl 'orange juice' (contains 0.25% w/v orange G, 15% w/v ficoll and 0.5M EDTA at pH7.0) and 8µl water. Samples were separated in parallel with pGem DNA markers (range 36-2,645bps, Promega) or Hae III øX174 markers (range 72-1353bp, IBI) by electrophoresis at 100V for 1-2h in 1 x TBE, viewed under UV light and photographed. Pure plasmid DNA appeared as two visible 'bands' of greater than 2kb (depending on plasmid size); one for the circular DNA and the other representing supercoiled DNA which migrates more rapidly through the gel due to its compact form.

3.6. Preparation of radiolabelled probes for Northern blot analysis

3.6.1. Preparation of DNA for labelling

Double stranded DNA for labelling was usually prepared by amplification of the cloned cDNA insert from a plasmid vector using polymerase chain reaction (PCR; Saiki *et al.*, 1988). This was achieved using pairs of T3 and T7 primers (Bluescript SK- vector; Stratagene, Cambridge, England) or SP6 and T7 primers (TA cloning vector; Invitrogen, Abington, England). The primers were used at a concentration of 0.5µM in a PCR reaction containing 100µM each dNTP (Pharmacia, Milton Keynes, England), Taq polymerase buffer (50mM KCl, 10mM Tris-HCl, 1.5mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X100) and 2.5U Taq polymerase (Promega). Plasmid DNA (100-500ng) containing the desired cDNA insert was used as a template for the reaction. Thirty-five cycles of amplification were performed with an annealing temperature of 45°C and a 1.5min extension at 72°C.

Amplification of the correct size DNA insert was checked by running 10µl of the PCR sample on an agarose gel with known DNA markers (see section 3.5.3). DNA was isolated from the PCR mix using Clontech ChromaSpin +TE-100 columns (Cambridge Bioscience, Cambridge, England) according to the manufacturers instructions.

3.6.2. Radiolabelling double stranded DNA

Double stranded DNA was radiolabelled using the random primer method (Feinberg & Vogelstein, 1983) and an Amersham 'Multiprime' kit according to the manufacturers instructions. Briefly, DNA (25-50ng) was denatured at 98°C for 5min. DNA was labelled with 50µCi of ^{32}P-[α]-dCTP in a reaction containing 5µl primer solution containing random hexanucleotides, 10mM each dATP, dGTP and dTTP, and 5µl reaction buffer containing Tris/HCl pH7.8, $MgCl_2$ and 2-mercaptoethanol. The reaction was catalysed by addition of 2U Klenow enzyme and incubation was for 30min at 37°C. Labelled DNA was denatured with 5N NaOH (100µl), neutralised with 1M Tris, pH7.6 (600µl) and 1N HCl (375µl) and added to the hybridisation mixture (section 3.7.2).

3.6.3. Synthesis of oligonucleotides

Antisense oligonucleotide for 18S ribosomal RNA (Chang et al., 1984) was used to check for even transfer of RNA on Northern blot. Oligonucleotides (17-24mers) were synthesised using phosphoramadite chemistry on a Model 381 DNA synthesiser (Applied Biosystems, Warrington, UK). Oligonucleotides were recovered into 1ml of pure concentrated ammonia, deprotected by incubating overnight at 65°C, recovered by two rounds of ethanol precipitation and resuspended in TE buffer (appendix I). The concentration of the oligonucleotides were estimated by spectroscopy at 260nm where an optical density of 1.0 is equal to a concentration of 20µg/ml.

3.6.4. Oligonucleotide labelling

Oligonucleotides were labelled at the 5' end using polynucleotide kinase (PNK). DNA was incubated with [γ - ^{32}P]ATP in a reaction mixture containing 50ng DNA, 30µCi [γ-^{32}P]ATP, 1 x kinase buffer (10 x buffer contains 0.5M Tris/HCl, 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine and 1mM EDTA) and 8U T4 PNK (Amersham). The reaction was allowed to continue at 37°C for 30 minutes. Unincorporated nucleotides were removed by passing the reaction mixture down a Clontech ChromaSpin +TE-10 spin column. The labelled oligonucleotide was then added directly to DNA hybridisation mix (section 3.7.2).

3.7. Northern blot analysis

3.7.1. RNA transfer to membrane

RNA separated on a denaturing gel (section 3.3.3) was transferred to nylon membrane (Hybond-N; Amersham) by capillary blotting as shown in Fig.2. Nylon membrane was prewetted with pure water followed by 20 x SSC before placing carefully on the gel. Transfer using 20 x SSC was allowed to continue for a minimum of 12h. After transfer the position of the gel wells were marked on the membrane and RNA was bound using a UV crosslinker.

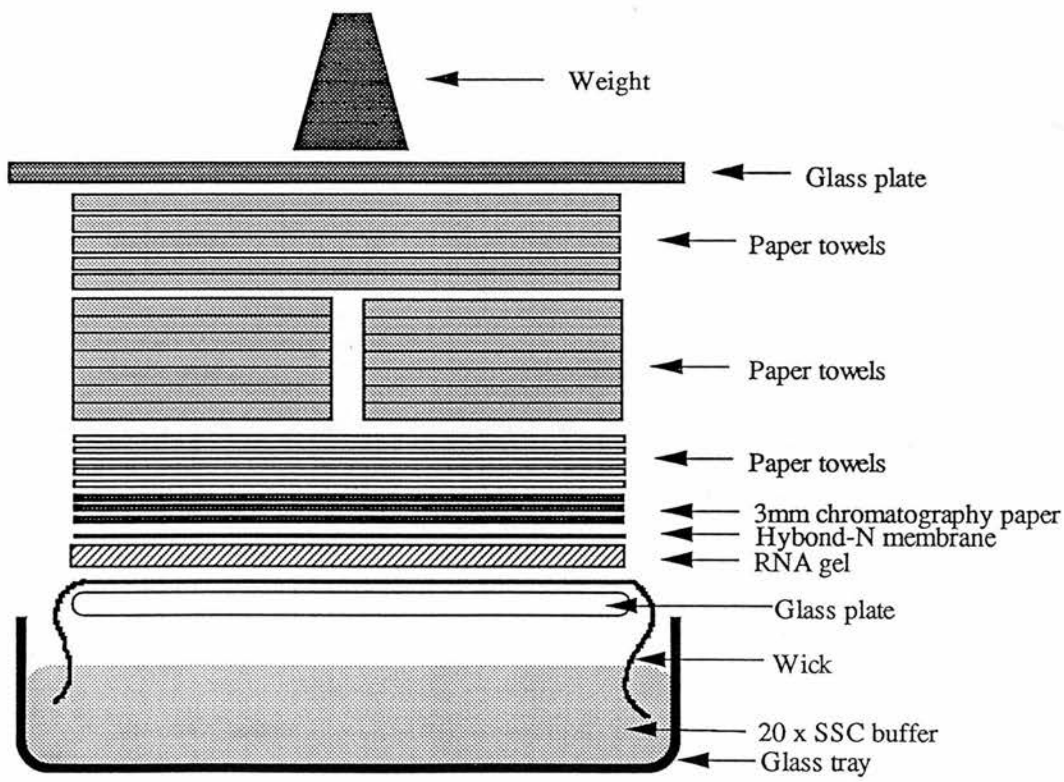


Figure 2. Northern blotting. Schematic drawing of the apparatus used for the transfer of RNA from a denaturing agarose gel to Hybond-N nylon membrane by cappillary action.

3.7.2. Hybridisation of radiolabelled probe to membrane

Double Stranded Probes. Membranes were prehybridised at 65°C for 2-4 hours in buffer containing 0.2M sodium phosphate pH7.2, 1mM EDTA, 1% BSA, 7% SDS and 15% formamide. Radiolabelled probe was added to the hybridisation mix at a final concentration of 0.5 - 1 x 10^6 cpm/ml buffer. Hybridisation was allowed to continue for 24-48 hours at 65°C.

Oligonucleotide Probes. Membranes were prehybridised at Tm of probe minus 10°C. Tm is the melting temperature of the probe dependent on the nucleotide content of the DNA and was calculated using the formula (4 x G+C) + (2 x A+T). Prehybridisation buffer contained 0.05% w/v BSA, 0.05% w/v polyvinylpyrrolidine, 0.05% w/v ficoll, 0.1% w/v SDS, 0.1% w/v sodium pyrophosphate, 5 x SSC and 100μg/ml sonicated salmon sperm DNA (Sigma). Labelled probe was added to the mix and hybridisation allowed to proceed for 24-48 hours.

3.7.3. Post-hybridisation washes

Double Stranded Probes. Membranes were washed with buffer containing 40mM sodium phosphate pH7.2, 1mM EDTA and 1% SDS. Washes were at 65°C for 2 x 30 minutes.

Oligonucleotide Probes. Membranes were washed with 4 x SSC at Tm minus 5°C for 2 x 30 minutes. For more stringent washes 1 x SSC, 0.5 x SSC or 0.1 x SSC were used.

3.7.4. Development of signal

After washing, membranes were air dried briefly and wrapped in clingfilm. The membrane was then exposed to X-ray film (XAR-5 or X-Omat S; Kodak) in cassettes with Dupont enhancing screens at -70°C. After a specific exposure time the signal was developed using LX 24 developer and fixed using FX 40 fixative according to the suppliers recommendations (both Kodak).

3.8. Probe preparation for *in situ* hybridisation

3.8.1. Template preparation

Plasmid DNA prepared as described previously (see section 3.5) was linearised in a reaction containing 1μg DNA, reaction buffer (contains 10-50mmol/l Tris/HCl, 5-10 mmol/l $MgCl_2$, 50-100mmol/l NaCl and 1mmol/l DTT, DTE or ß-mercaptoethanol), 10U restriction enzyme and pure water to a volume of 40μl. The enzyme used was dependent on the plasmid vector being digested and the direction of synthesis of the riboprobe. The reaction was incubated at 37°C for 1-2h. Thereafter, 3μl of reaction mix was run on a minigel (0.8% agarose) and compared to an uncut sample of the same plasmid run in a parallel lane to test efficiency of digestion. Digested DNA was then extracted once with Tris-buffered phenol:chloroform and precipitated with 1/10th volume 3M sodium acetate pH5.5 and 2.5 volumes absolute ethanol overnight at -20°C. Linearised DNA was pelleted, dried and resuspended in 10µl pure water.

3.8.2. Preparation of radiolabelled riboprobe

Synthesis of riboprobes was carried out using 1µg linearised template in a reaction mix containing 10mM DTT, 20U RNase inhibitor, 1mM each rATP, rCTP and rGTP, transcription buffer (1 x transcription buffer contains 40mM Tris/HCl, pH7.9, 6mM $MgCl_2$, 2mM spermidine and 10mM NaCl) and 50µCi ^{35}S-UTP. The reaction was catalysed by addition of 30U of the appropriate RNA polymerase (T3, T7 or SP6) and incubated at 37°C for approximately 1.5h. The DNA template was then removed by digestion with 40U RNase-free DNase for 15min at 37°C. Enzymes and salts were removed by two rounds of phenol/chloroform extraction, RNA was precipitated with sodium acetate and ethanol overnight at -70°C and recovered by centrifugation as above.

Radiolabelled RNA was air dried, resuspended in 50µl pure water and the activity of 2 x 1µl aliquots was determined by liquid scintillation spectroscopy. The average activity of the two samples was calculated and the volume of probe necessary to give 1 x 10^6 cpm was determined.

3.8.3. Preparation of digoxigenin labelled riboprobe

Synthesis of a digoxigenin (DIG) labelled riboprobe was essentially the same as for the radiolabelled riboprobes above except that 1µg template was incubated with 1mM each rATP, rCTP and rGTP, 0.65mM rUTP and 0.35mM DIG-labelled UTP (Boehringer Mannheim) in 1x transcription buffer with 20U RNase inhibitor and 40U RNA polymerase for at least 2h at 37°C. The template DNA was again removed by DNase treatment, the RNA precipitated and resuspended in 50µl pure water.

Concentration of the labelled RNA was determined by comparison with known amounts of labelled control RNA (Boehringer Mannheim). Aliquots of DIG-labelled riboprobe (1µl) were spotted onto nylon membrane (Hybond-N, Amersham) in parallel with 1µl labelled control RNA of different concentrations. RNA was fixed to the membrane by UV crosslinking. Visualisation of the labelled RNA was as for development of hybridised DIG labelled RNA for *in situ* hybridisation (see section 3.10.2) with the exception that the antibody was used at a dilution of 1:1500.

3.9. Radioactive *in situ* hybridisation

3.9.1. Pretreatment of tissue

Paraffin wax embedded tissue was cleared in histoclear for 15min and rehydrated in a series of alcohols with decreasing concentrations. Tissue was placed in 0.2N HCl for 20min followed by two 5min washes in distilled water. Sections were then incubated in 2µg/ml proteinase K (Sigma) in buffer containing 20mM Tris/HCl pH7.4 and 50mM EDTA at 37°C for 20min followed by 0.2% glycine at 4°C for 10min. Sections were washed briefly in 0.1M triethanolamine (TEA) pH8.0 and acetylated in 0.25% acetic anhydride in 0.1M TEA pH8.0 for 10min. Finally, sections were rinsed in distilled water and prehybridised in buffer containing 4 x STE (1 x STE contains 150mM NaCl, 2.5mM Tris and 0.25mM EDTA), 1 x Denhart's solution (50 x Denhart's contains 5g BSA, 5g polyvinylpyrrolidine and 5g ficoll in 500ml solution), 10mM DTT, 125µg/ml salmon sperm DNA, 125µg/ml yeast transfer RNA and 50% deionised formamide for 2-4h at probe Tm-25°C.

3.9.2. Hybridisation of probe to tissue

Hybridisation was continued overnight with a probe concentration of 1 x 10^6 cpm in 40µl buffer/slide. Hybridisation buffer was prehybridisation buffer with 10% dextran sulphate. The incubation was carried out beneath coverslips prepared from gel bond film (Flowgen Instruments Ltd.) in a humidified chamber at Tm-25°C where Tm was calculated individually for each probe according to the equation:

$$Tm = 79.8 + 58.4\,(F_{GC}) + 11.8\,(F_{GC})^2 + 18.5\,\log(M) - 820/L - 0.35\,(\%F) - (\%M)$$

where:

F_{GC} = mole fraction of GC content of probe, usually about 0.45

M = monovalent cation concentration (molarity of salt in hybridisation buffer)

L = length of duplexes formed during hybridisation (probe length)

%F = percentage of formamide in buffer

3.9.3. Post hybridisation washes

After incubation sections were washed in two changes of 4 x SSC for 5min each to remove the coverslip and treated with RNase A (Sigma) at a concentration of 20µg/ml in 0.5M NaCl, 0.1M Tris pH8.0 and 1mM EDTA for 30min at 37°C. Sections were washed in RNase buffer alone for 30min at 37°C followed by two washes in 2 x SSC at room temperature and 45°C for 30min each and a final wash in 0.5 x SSC at room temperature for 30min. More stringent washes were carried out when appropriate for particular riboprobes and are mentioned in the relevant chapters.

3.9.4. Development of *in situ* hybridisation

After post hybridisation washes sections were dehydrated through alcohols. Slides were warmed to 45°C and dipped in prewarmed NTB2 emulsion (Kodak) at 45°C in the dark. Emulsion coated slides were stored in a humidified, lightproof box overnight before transfer to a polyacetyl black trough (lightproof; Lamb's laboratory supplies, London, England) with silica gel. and stored at 4°C for a minimum of 7 days.

Silver grains formed by reaction of the hybridised, radiolabelled probe with the emulsion were developed using Kodak D19 developer as follows. Slides were placed in developer cooled to 14°C for 4min, followed by a brief wash in pure water and grains were fixed by incubation for 5min in Kodak unifix at 14°C. Sections were washed in water, stained with haematoxylin, dehydrated and mounted in Eukitt (O.Kindler GmbH & Co. supplied by Laboratory Sales Ltd., Rochdale, England).

Slides were analysed under dark field using either BIOMED or LABORLUX 12 microscopes (Leitz, Wetzlar, Germany) or the Zeiss Photomicroscope III (Hertfordshire, England) to visualise the silver grains indicating areas of hybridisation. Bright field microscopy was used to examine the morphology of the testis sections and to allow identification of the stages of the spermatogenic cycle.

3.10. Non-radioactive *in situ* hybridisation

3.10.1. Pretreatment and hybridisation of tissue

Pretreatment of tissue was essentially as for radioactive *in situ* hybridisation with a few modifications. Proteinase K treatment was for 40min in a concentration of 2µg/ml. Prehybridisation was for 2-4h as before but buffer did not contain DTT. The amount of formamide in the buffer was varied according to the probe used and the concentration is specified in the relevant chapters.

Hybridisation was carried out with a labelled riboprobe at a concentration of 200ng probe/ml buffer. All other hybridisation conditions were as for radioactive *in situ* hybridisation.

Post hybridisation washes were two changes of 4 x SSC for 5min each to remove the coverslip and then RNase A at a concentration of 20µg/ml in buffer for 30min at 37°C. Slides were washed in two changes of 2xSSC at room temperature followed by 15min in 0.1xSSC / 30% formamide at 40°C.

3.10.2. Development of *in situ* hybridisation

After washing slides were equilibrated in 0.01M Tris buffered saline pH7.5 (TBS) for 2min before incubating in normal sheep serum diluted 1:5 with TBS for 30min to reduce background staining. The slides were washed briefly in TBS and antibody was added. This was an anti-DIG antibody conjugated to alkaline phosphatase. The antibody was diluted 1:300 in TBS and incubated with the sections for 2h. Excess antibody was removed with 2 x 15min washes in TBS and sections were equilibrated in buffer containing 0.1M Tris/HCl, pH9.5, 0.1M NaCl and 50mM $MgCl_2$ for 2min before development of the colour reaction. Substrates for the alkaline phosphatase were nitrobluetetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate). Substrate solution contained 337.5μg/ml NBT and 175μg/ml X-phosphate and was incubated on the sections at room temperature in the dark until colour developed (usually overnight). Sections were washed in 0.1M Tris/HCl, pH8.0, 1mM EDTA and stained with haematoxylin before dehydrating and mounting.

3.11. Immunohistochemistry

3.11.1. Tissue pretreatment and primary antibody

Tissue prepared as described in section 4 of this chapter was cleared in histoclear for approximately 10min and hydrated in decreasing concentrations of ethanol. The sections were placed in 3% hydrogen peroxide in pure methanol for 30min to reduce background staining. This pretreatment was only included if the detection system involved the enzyme horse radish peroxidase; it was not necessary for the alkaline phosphatase detection system. Slides were washed in TBS pH7.6 for 5min and the tissue blocked with normal rat serum diluted 1:5 in TBS for 10min at room temperature.

CP-2 primary antibody was used undiluted after adsorption and PEG concentration while SGP-1 was used at a dilution of 1:1000 (diluted in 1:5 normal swine serum: TBS). Tissue was incubated with the antibody overnight at room temperature. Preimmune rabbit serum at a dilution of 1:100 or 1:1000 in TBS was used as a negative control. Excess antibody was removed by two 5min washes with TBS.

3.11.2. Secondary antibody and detection system

Horse radish peroxidase (HRP) detection system. The secondary antibody for the HRP detection system was biotinylated swine anti-rabbit immunoglobulins (SARB; Sigma). SARB was diluted 1:500 in TBS and incubated on the tissue sections for 30min. Excess antibody was again removed by 2 x 5min washes with TBS. Sections were incubated with an AB-HRP complex (Dakopatts, Glostrup, Denmark) for 30min. This is a solution of avidin and biotinylated-HRP which serves to amplify the signal obtained from the primary antibody. The solution is prepared according to the suppliers instructions in 0.05M Tris/HCl pH7.6 at least 20min before use. Excess AB complex was removed by 2 x 5min washes in TBS and bound antibody visualised with a solution of 225μM diaminobenzidine in 0.05M Tris/HCl, pH7.6 containing 0.01% hydrogen peroxide.

Alkaline phosphatase (AP) detection system. The secondary antibody for this detection system was sheep anti-rabbit immunoglobulins (SAR; Sigma). SAR was diluted 1:30 in TBS and incubated on the sections for 30min. This was followed by 2 x 5min washes with TBS. Sections were incubated for 30min in rabbit phosphatase anti-phosphatase (Dakopatts) diluted 1:60 in TBS followed by 2 x 5min washes in TBS. Sections were equilibrated in buffer containing 0.1M Tris, pH9.5, 0.1M NaCl and 50mM $MgCl_2$, for 2min. Substrates for the alkaline phosphatase were nitrobluetetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate) and the detection was carried out as for non-radioactive *in situ* hybridisation (section 10.2).

After development of the colour reaction sections were washed in water, stained with haematoxylin, dehydrated and mounted with coverslips.

3.12. DNA sequencing

3.12.1. Manual sequencing reactions

DNA was sequenced using the dideoxy chain termination method and the Sequenase enzyme (United States Biochemical Corporation, supplied by Cambridge BioScience). Primers directed against sequences on the templates were synthesised as described in section 3.6.3.

3.12.1.1. Annealing reaction

PCR amplified DNA templates. PCR amplified DNA (see section 3.6.1) was purified using Clontech Chroma Spin +TE-100 columns. DNA (250-750bp) was mixed with 300ng sequencing primer in a total reaction volume of 10μl containing 1μl DMSO and 2μl 5x Sequenase reaction buffer (containing 200mM Tris/HCl, pH7.5, 100mM $MgCl_2$ and 250mM NaCl), incubated at 100°C for 2min and then placed on ice.

Plasmid DNA templates. Plasmid DNA was prepared from bacterial cultures by the alkaline lysis method previously described (section 3.5.2) and 3-5μg DNA was used in the sequencing reaction. Direct sequencing of plasmid DNA was carried out using the alkaline denaturation method. DNA was denatured in 0.2M NaOH for 5min at room temperature, neutralised with 0.4 volumes 5M ammonium acetate, pH7.5 and the DNA precipitated with 4 volumes absolute ethanol at -70°C for 15min. DNA was pelleted by centrifugation, the supernatant discarded and the DNA resuspended in 7μl water. Annealing with primer in the presence of Sequenase reaction buffer was achieved by heating the reaction mixture at 65°C for 2min and then allowing the reaction to cool slowly to below 35°C (approximately 30min).

Reactions were then continued following the same protocol for sequencing of both PCR amplified and plasmid DNA templates.

3.12.1.2. Labelling, extension and termination reactions

Labelling of DNA was carried out by adding 1μl 0.1M DTT, 2μl dGTP labelling mix (diluted 1:15 with TE buffer; mix contains 7.5μM each dGTP, dCTP and dTTP), 0.5μl ^{35}S-dATP and 1.5μl Sequenase enzyme (diluted 1:6 in 10mM Tris/HCl, pH7.5, 5mM DTT and 0.5mg/ml BSA) to the annealing mix and incubating at room temperature for 5min.

The extension of labelled DNA was terminated by adding 3.2μl labelling reaction to 2μl of each of the four termination mixes, ddGTP, ddATP, ddCTP and ddTTP, which were prewarmed at 37°C in separate tubes. Each termination mix contained 80μM each of the four deoxyribonucleotides, 50mM NaCl and 8μM of the appropriate dideoxyribonucleotide. Incubations were continued at 37°C for a further 10min and the reactions were stopped by adding 4μl stop dye containing 95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF.

3.12.2. Preparation of manual sequencing gel

A sequencing gel composed of 9% acrylamide (30%, bis 19:1) and 1.5 x TBE in 12M urea was prepared on BioRad Sequi-Gen Sequencing apparatus. Three gel mixes were prepared containing the ingredients outlined in Table 1.

Table 1. Gel mixes for manual sequencing.

Reagent		**Mix**	
	Top	Bottom	Base
Urea	27.6g	9.2g	18.4g
Acrylamide	12ml	4ml	8ml
10 x TBE	6ml	5ml	10ml
Water	20ml	2ml	4ml

Urea was melted by heating mixes in the microwave for 2 minutes at 30% power prior to the addition of 100μl each of 10% ammonium persulphate and TEMED to the base mixture which was poured into the casting tray. The bottom of the assembled gel apparatus was placed in the casting tray and the base mix was allowed to set to seal the bottom of the gel plates.

The 'top' and 'bottom' mixes were filtered using a 0.45μM Millipore filter and a syringe. Ammonium persulphate and TEMED were added (60μl and 20μl of each to top and bottom, respectively) and the gel was poured immediately as follows. Using a 25ml pipette, 7.5mls of top mix followed by 6.5mls of bottom mix were drawn up into the pipette followed by a couple of bubbles to mix. This was poured down the middle of the gel plates. The remainder of the top mix was then poured between the gel plates until the top was reached. The gel comb was placed, upside down, between the gel plates at the top of the apparatus to form a single long well. The gel was left to polymerise at room temperature for approximately 1h. The comb was removed from the gel, the well washed out with 1 x TBE and the gel was prerun in 1xTBE at 1800V for about 30 minutes. The comb was replaced in the gel with the sharks teeth protruding a couple of millimetres into the gel forming wells for sample loading. Sequencing reactions were heated at 75°C for 3min before loading 4μl of each sample onto the gel. This was then run for 2-3 hours at 1800 volts or overnight at 600V or 900V.

The gel was removed from between the glass plates, vacuum dried onto 3mm filter paper for approximately 2h at 80°C and exposed to X-Omat AR film (Kodak) overnight at room temperature.

3.12.3. Automatic sequencing

Automatic sequencing reactions were carried out using the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and were run on the Applied Biosystems Model 373A DNA sequencing system.

3.12.3.1. Sequencing reactions

A reaction premix was prepared containing the equivalent of 4μl 5x TACS buffer (400mM Tris/HCl, 10mM $MgCl_2$ and 100mM $(NH_4)_2SO_4$ pH9.0), 1μl dNTP mix (containing 750μM dITP and 150μM each dATP, dTTP and dCTP), 1μl each ddATP, ddTTP, ddCTP and ddGTP terminators and 0.5-1μl AmpliTaq DNA polymerase for each sequencing reaction to be performed. For sequencing of PCR products 1μl DMSO was also added. A total of 11.5μl (PCR template) or 9.5μl (plasmid DNA template) of the reaction premix was added to separate tubes containing 3.2 pmol primer and 5μl template DNA, the total volume adjusted to 20μl with water and overlaid with mineral oil.

Tubes were placed in a Perkin Elmer Cetus Model 480 preheated to 96°C and 25 cycles of thermal cycling carried out as follows; rapid thermal ramp to 96°C, 96°C for 30s, rapid thermal ramp to 50°C, 50°C for 15secs, rapid thermal ramp to 60°C and 60°C for 4min.

On completion of the sequencing reaction, the samples were extracted twice with water saturated phenol:chloroform and precipitated with 15μl 2M sodium acetate, pH4.5 and 300μl cold absolute ethanol to remove unincorporated labelled terminators. Labelled DNA was pelleted by centrifugation, the supernatant discarded and the DNA washed using 75% ethanol, air dried and resuspended in formamide/EDTA buffer (5μl formamide, 1μl 50mM EDTA, pH8.0) just before loading onto the gel.

3.12.3.2. Automatic sequencing gel

A sequencing gel mix containing 50g urea, 15ml 40% acrylamide and water made up to a final volume of 80ml was prepared. Amberlite resin was added to remove acrylamide free acid and the mixture was stirred and heated until the urea had dissolved, the solution was filtered and 10ml 10x TBE added. The gel mix was polymerised using 45μl TEMED and 500μl 10% ammonium persulphate and poured carefully between clean glass plates.

The gel was prerun for 10-15min at 500V. Samples resuspended in buffer were heated to 95°C for 5min before loading onto the gel. The gel was run overnight using the automatic data collection and analysis programs.

3.13 Identification of the stages of the spermatogenic cycle

Periodic acid-Schiffs base staining of sectioned rat testis (described in section 3.4.3) was used as an aid for definition of the stages of the seminiferous tubules. The fourteen stages of the spermatogenic cycle were defined on the basis of;

1. germ cell complement of the seminiferous tubules
2. presence of mitotic cells
3. size and shape of the spermatid acrosome
4. position of elongate spermatids within the seminiferous epithelium.

4. Preliminary Investigations into the Germ Cell Regulation of Sertoli cell mRNAs

4.1. Introduction

Spermatogenesis is controlled by the gonadotrophins produced in the pituitary gland (Pierce & Parsons, 1981) and by LH-stimulated testosterone synthesis in the Leydig cell (Rommerts & Cooke, 1988). Sertoli cells are the only somatic cell type within the seminiferous epithelium and as such play a pivotal role in the support of spermatogenesis. They are known to provide the germ cells with the factors necessary for their development and progression through the spermatogenic cycle. Sertoli cells synthesise and secrete numerous proteins many of which are believed to be important in the control of spermatogenesis. The regulation of Sertoli cell protein synthesis is partly under hormonal control but it is becoming increasingly apparent that cellular interactions in the testis are also important in modulating Sertoli cell activity. Whilst the seminiferous tubules at each stage of the spermatogenic cycle are exposed to the same circulating hormone levels, the Sertoli cells show a distinct cyclic function (Parvinen, 1993). This cyclicity of function is thought to be regulated in part by the changing complement of germ cells present at each stage of the spermatogenic cycle. Studies both *in vivo* and *in vitro* have shown that several proteins synthesised by Sertoli cells are regulated by the presence of, or the secretion of factor/s by, specific germ cells (see Sharpe, 1993).

The aim of this study was to investigate potential germ cell regulation of known Sertoli cell proteins at the level of gene transcription. The identification of proteins suitable for study was based on the observation of a cyclical pattern of secretion or previous reports of possible germ cell regulation. The Sertoli cell products studied were α-inhibin, ABP, CP-2, CRBP, cystatin C, transferrin, SGP-1 and SGP-2.

Inhibin is a protein produced by Sertoli cells *in vivo* which negatively regulates the secretion of FSH from the pituitary gland. Stage specific expression of inhibin-α mRNA was shown by RNA dot blot hybridisation of microdissected staged seminiferous tubules; levels were highest at stages XIII-I and lowest at stages VII-VIII of the cycle (Bhasin *et al.*, 1989). Possible germ cell regulation of α-inhibin production by the Sertoli cell has been shown. Various studies either using the addition of germ cells to immature Sertoli cells *in vitro* or the depletion of germ cells from adult testis *in vivo* have shown effects of spermatocytes, and early and late spermatids on immunoreactive inhibin secretion (Pineau *et al.*, 1990; Allenby *et al.*, 1991). Some of the results obtained are conflicting and this may be due to differences in experimental procedures. I therefore hoped to clarify the situation by the study of α-inhibin mRNA using an *in vivo* model of germ cell depletion described in chapter 3.

Androgen binding protein (ABP) is secreted by the Sertoli cell in a stage dependent manner with maximal secretion occurring at stage VII (Parvinen, 1982). This protein binds testosterone and DHT with high affinity but its precise function in the testis is unclear. Again, several studies have indicated that ABP synthesis may be regulated by specific germ cells. *In vivo* studies have shown that the depletion of late spermatids from the seminiferous epithelium results in a decrease in ABP secretion (Bartlett *et al.*, 1988; Pinon-Lataillade *et al.*, 1988). However, it is not known at which level this regulation occurs and so expression of ABP mRNA on depletion of different germ cells from the testis was studied to see if regulation was at the level of gene transcription.

Cyclic protein-2 (CP-2) was first identified on 2D gels of secreted proteins from isolated staged seminiferous tubules and was shown to be secreted in a cyclical manner with maximal secretion at stages VI and VII of the spermatogenic cycle (Wright *et al.*, 1983). The function of the protein within the seminiferous epithelium is unknown. A cDNA coding for CP-2 was isolated recently and was shown to be homologous to the proenzyme form of cathepsin L, a cysteine protease (Erickson-Lawrence *et al.*, 1991). Expression of the mRNA for CP-2 was shown to be stage specific with maximal expression occurring at stages VI and VII as for the protein secretion. However, no studies on possible germ cell regulation of the protein or its mRNA had been undertaken although its marked stage specificity suggests this possibility.

Vitamin A is known to be essential for mammalian spermatogenesis since when an animal is deprived of vitamin A the seminiferous epithelium regresses and spermatogenesis is arrested (Wolback & Howe, 1925). This can be reversed by administration of retinol to the animal (Howell *et al.*, 1963). Sertoli cells have been shown to synthesise a retinol binding protein which may be involved in the transport of retinol in the testis (Davis & Ong, 1992). Cellular retinol binding protein (CRBP) mRNA has been detected in the testis by *in situ* hybridisation, has been localised mainly to the Sertoli cells and is expressed maximally at stages IX-XIII of the spermatogenic cycle (Rajan *et al.*, 1990a). This cyclicity of mRNA expression is reflected by changes in protein synthesis as seen by immunohistochemistry, with the strongest staining observed at stages XII-XIV of the cycle (Porter *et al.*, 1985). This pattern of mRNA expression and protein synthesis was of interest since it was unlike that seen for the Sertoli cell products ABP and CP-2 which were not secreted at the later stages of the spermatogenic cycle. This could indicate that regulation of production of these Sertoli cell proteins occurs by different mechanisms or is under the control of a different germ cell population.

Cystatin C is a member of the cystatin superfamily of cysteine proteinase inhibitors (Barrett, 1987). It is a low molecular weight alkaline protein of 120 amino acids and was first isolated from urine of patients with renal tubular disorders (Cejka & Fleischman, 1973). There are several forms of the protein with a native form of 13kDa and a glycosylated form of approximately 20kDa. The protein has been shown to bind cathepsin L with very high affinity and to block its biological action (Barrett, 1987). Cystatin C has a very wide tissue distribution but analysis of the content of various tissues and biological fluids in the rat showed highest levels of the protein in seminal vesicles (Tavera *et al.*, 1990). Sertoli cells isolated from 18 day old rats were shown to produce cystatin C in culture (Esnard *et al.*, 1992). The presence of cystatin C in the testis and the male reproductive tract and its ability to block the actions of proteases suggests that it may have a role to play in the movement of spermatids in the seminiferous epithelium and/or spermatozoa in the reproductive tract. It was therefore of interest to study the exact distribution of cystatin C in the testis and the possible regulation of its production by germ cells.

The iron binding protein transferrin is known to be synthesised by Sertoli cells (Skinner & Griswold, 1980). It has been proposed that its function in the Sertoli cell is to bind iron and transport it to the germ cells where it is necessary for their development. The expression of transferrin mRNA has been reported to occur in a stage-dependent manner (Morales *et al.*, 1987). Numerous studies carried out *in vitro* using immature Sertoli cells in culture have shown that germ cells can stimulate transferrin secretion (Le Magueresse *et al.*, 1988; Onoda & Djakiew, 1990) and the level of expression of its mRNA (Stallard & Griswold, 1990). However the use of immature Sertoli cells in culture does not necessarily give a true indication of the regulation which will occur in the adult animal *in vivo*. Therefore I used an *in vivo* model of MAA-induced germ cell depletion in the adult rat to study possible germ cell regulation of transferrin production by Sertoli cells.

The sulphated glycoproteins (SGP-1 and SGP-2) have been studied quite extensively in the testis but their functions remain largely unknown. They are secreted in high amounts by the seminiferous tubules (Sylvester *et al.*, 1984; 1989) and their mRNAs are heavily expressed in Sertoli cells (Morales *et al.*, 1989). It has been shown that production of SGP-1 and SGP-2 by adult Sertoli cells is not regulated by hormones (Roberts *et al.*, 1991; 1992) or by the presence of germ cells (Stallard & Griswold, 1990; Grima *et al.*, 1992). These proteins were therefore included in the present studies to act as negative controls so that germ cell regulation observed for any other Sertoli cell products could be shown to be a true effect of germ cell depletion and not an artefact of the experimental system employed.

All of the Sertoli cell proteins analysed in this study have been described previously in the literature and their cDNA sequences have been published. The model used involved germ cell depletion of adult rat testes using the testicular toxicant methoxyacetic acid (MAA). Treatment of rats with an oral dose of 650mg/kg MAA results in the depletion of pachytene spermatocytes at all stages of the spermatogenic cycle with the exception of early to mid stage VII (see chapter 3, section 2.2). Spermatogenesis then proceeds with normal kinetics such that at selected time points after MAA treatment round and then elongate spermatids are selectively absent from the testis because of maturation depletion. RNA was isolated from total testis tissue (see chapter 3, section 3) and tissue was perfusion fixed (see chapter 3, section 4) at times when either pachytene spermatocytes, round spermatids or elongate spermatids were depleted from the seminiferous tubules. Northern blot hybridisation (see chapter 3, sections 6 & 7) and *in situ* hybridisation studies (see chapter 3, sections 8, 9 & 10) were then performed to detect any germ cell regulation of the selected Sertoli cell proteins.

4.2. Experimental Procedures

4.2.1 Preparation of cDNA's specific for the Sertoli cell mRNA's

Transferrin. A 688bp cDNA for the 3' region of rat transferrin was obtained from Dr. Steve Sylvester (University of Washington, Pullman, Washington, USA; Huggenvik *et al.*, 1987). The cDNA was contained in the SP64 plasmid vector (Promega). This was transformed into DH5α cells (Gibco BRL, Paisley, Scotland) and glycerol stocks were taken to provide a permanent source of the plasmid.

Cellular Retinol Binding Protein (CRBP). A 700bp cDNA for rat CRBP contained in the plasmid vector pGem-3 (Promega) was obtained from Dr. D. S. Goodman, Columbia University, New York (Sherman et al., 1987). This was transformed by heat shock into DH5α cells according to the manufacturers instructions.

Androgen Binding Protein (ABP). A 1400bp cDNA specific for rat ABP contained in the SP65 vector was obtained from Dr. David Joseph (University of North Carolina, Chapel Hill, USA; Joseph *et al.*, 1987). The plasmid was digested with Eco RI to remove the insert and due to internal cleavage of the cDNA two fragments of 650 and 750bps were released. The 750bp fragment was subcloned into the Eco RI site of the pSP6/T3 vector (Gibco) and transformed into competent E.coli HB101 cells (Gibco) as above.

Cyclic Protein-2 (CP-2). Oligonucleotide primers were selected to amplify from base 6 to base 499 of the published sequence of CP-2 (Erickson-Lawrence *et al.*, 1991). The sequences were as follows, 5' primer (sense strand) 5'AGG CAG ATA GTG AAT GGC TAT CG3' (bases 6-28) and 3' primer (antisense strand) 5'T GGC TTG CAT CCA TGG CAA CAG3' (bases 477-499). The primers had the sites for Eco RI (TCG GAA TTC) and Hind III (TCG AAG CTT), respectively added to their 5' ends. Primers were synthesised on a Model 381 oligonucleotide synthesiser and recovered as described in section 3.6.3.

These primers were used to amplify a partial cDNA clone for CP-2 by polymerase chain reaction from a pool of rat testis cDNAs prepared by random priming of mRNAs using standard methods (Okayama & Berg, 1982). The PCR reaction mix was as described in chapter 3. Thirty-five cycles of amplification were performed with an annealing temperature of 61°C and a 1 min extension at 72°C. A single 493bp cDNA was amplified and purified using the Qiagen PCR purification kit (Diagen, Germany). To confirm that it was identical to the published sequence the purified cDNA was sequenced by chain termination using Sequenase and methods described in chapter 3. The isolated cDNA was subcloned into the Bluescript SK^- vector as described in section 4.2.2.

Sulphated Glycoprotein-1 (SGP-1). Oligonucleotide primers were selected to amplify from base 598 to base 1146 of the published sequence of SGP-1 (Collard *et al.*, 1988). The oligonucleotide sequences were, 5' primer (sense strand) 5'AAG GCT AAC GAG GAC GTC TG3' (bases 598-617) and 3' primer (antisense strand) 5'TG CAT GAG GAC GTC CAA CAG3' (bases 1127-1146). The cDNA was amplified by reverse transcriptase PCR from rat testis mRNA. The reverse transcription reaction mix contained 1x r*Tth* reverse transcriptase buffer (10 x contains 100mM Tris/HCl, pH8.3 and 900mM KCl), 1mM $MnCl_2$, 200μM each dNTP, 5U r*Tth* reverse transcriptase (Perkin Elmer Cetus, Conneticut, USA), 0.75μM 3' primer and 250ng testis mRNA. The reaction was incubated at 65°C for 3min followed by annealing at 50°C for 10min and an extension time of 5min at 70°C. The synthesised cDNA was amplified in a normal PCR reaction. To the reverse transcription reaction mix was added (given as final concentrations) 1x chelating buffer (10 x contains 50% glycerol, 100mM Tris/HCl, pH8.3, 1M KCl, 7.5mM EGTA and 0.5% Tween 20), 1.875μM $MgCl_2$, 0.15μM 5' primer and H_2O to a final volume of 100μl. PCR was 35 cycles of 94°C for 1min (melting), 50°C for 1min (annealing) and 72°C for 1.5min (extension). The amplified cDNA was isolated for sequencing and was subcloned into the TA cloning vector (pCR II, InVitrogen, Abingdon, Oxon, UK.) according to the manufacturers instructions (see section 4.2.2).

Sulphated Glycoprotein-2 (SGP-2). Oligonucleotide primers were selected to amplify from base 343 to base 779 of the published sequence of SGP-2 (Collard & Griswold, 1987). The sequences were as follows, 5' primer (sense strand) 5'TGC CTG AAG CAC ACC TGC AT3' (bases 343-362) and 3' primer (antisense strand) 5'CTG GAC GTC CAT GGC CTG3' (bases 762-779). Using these primers a 438bp cDNA for SGP-2 was amplified by PCR from a plasmid containing an 1800bp SGP-2 cDNA insert, obtained from Dr. S. Sylvester (University of Washington), in the standard PCR reaction mix previously described (see chapter 3, section 6.1). Thirty cycles of amplification were performed with an annealing temperature of 55°C and a 1 min extension at 72°C. The amplified cDNA was purified, sequenced as before and subcloned into pCR II (see section 4.2.2).

Cystatin C. Oligonucleotide primers were selected to amplify from base 99 to base 313 of the published sequence of cystatin C (Cole *et al.*, 1989). The sequences were as follows, 5' primer (sense strand) 5'GCG TTG GAC TTC GCC GTA3'(bases 99-116) and 3' primer (antisense strand) 5'CAG AGT GCC TTC CTC ATC A3' (bases 296-313). The primers had the sites for Eco RI and Hind III, respectively added to their 5' ends. A partial cDNA for rat cystatin C was amplified from a pool of rat testis cDNAs using standard PCR conditions. Thirty five cycles of amplification were carried out with an annealing temperature of 57°C and a 1min extension at 72°C. The amplified cDNA was purified, sequenced and subcloned into the Bluescript vector (section 4.2.2).

Alpha inhibin. Oligonucleotides were selected to amplify from base 945 to base 1375 of the published sequence of α-inhibin (Woodruff *et al.*, 1987). The sequences were 5' primer (sense strand), 5'GGC TCG ACG TTC AGC TC3' (bases 945-961) and 3' primer (antisense strand), 5'AGG AGG ACA CGA GGT GCT3' (bases 1358-1375). The primers had the sites for Eco RI and Hind III, respectively added to their 5' ends. A 432bp cDNA was amplified by PCR from a 1333bp rat α-inhibin cDNA obtained from Dr. F. Esch at The Salk Institute, La Jolla, California. Twenty five cycles of amplification were carried out at an annealing temperature of 50°C and an extension time of 72°C for 1min. The amplified cDNA was purified and sequenced before cloning into Bluescript SK- (section 4.2.2).

4.2.2 Subcloning of cDNA's

Subcloning into the Bluescript vector. Purified, amplified cDNA was digested with Eco RI and Hind III to introduce cohesive ends. The small end fragments of DNA released by digestion were removed by passing down a Clontech Chroma Spin +TE-100 column. The cDNA was ligated into Bluescript SK$^-$ (Stratagene) which had previously been digested with Eco RI and Hind III and purified to remove the portion of vector released. The vector was purified by electrophoresis through a 0.8% Nusieve agarose gel (Stratech Scientific Ltd., Luton, UK) in 1 x TAE buffer (appendix I) followed by DNA extraction using the Geneclean II kit (Bio 101, Inc., California, USA) according to the manufacturers instructions. Briefly, the gel slice containing the isolated cut vector was melted in 3 volumes of a NaI solution at 50°C for 5min. Approximately 10µl GLASSMILK suspension was added and the mix cooled on ice for 5min to allow DNA binding to the silica matrix. The GLASSMILK/DNA complex was pelleted by brief centrifugation and the pellet washed 3 times in ethanol wash buffer. DNA was finally eluted by resuspension of the pellet in a small volume of water, incubation at 50°C for 3min and centrifugation to allow removal of the supernatant containing the plasmid DNA. Ligation of cDNA to cut vector was carried out with 100ng cDNA and 50ng linearised vector in a reaction containing 1 x ligation buffer (10 x ligation buffer contained 300mM Tris/HCl, pH7.8, 100mM $MgCl_2$, 100mM DTT and 10mM ATP), 1mM ATP, and 1U T4 DNA ligase (Promega) in a total volume of 10µl. The reaction mix was incubated at 4°C for 16-20h.

Subcloning into the TA vector. The amplified cDNA was subcloned into the pCR^{TM} II vector using the TA cloning system (InVitrogen) according to the manufacturers instructions. Amplified cDNA was ligated directly from the PCR reaction mix without the requirement for further purification at a 1:1 molar ratio of vector to PCR insert. Briefly, approximately 8ng cDNA insert was ligated into 50ng vector in a reaction mix containing 1 x ligation buffer and 1µl T4 DNA ligase in a total reaction volume of 10µl. The reaction was incubated at 12°C overnight and the plasmids were transformed into competent E.coli cells (INVαF') by heat shock (see below).

4.2.3 Transformation of E.coli with plasmids

Bluescript vector into XL1-blue cells. Bluescript plasmids were transformed into the E. coli bacterial strain XL1-blue. LB-broth (appendix I) containing 0.2% maltose and 10mM $MgSO_4$ was inoculated with XL1-blue from a glycerol stock and incubated overnight shaking at 200rpm at 37°C until the cells reached log phase, ie. the OD at

600nm of the cell suspension was 0·6. The cell suspension was centrifuged at 1600g and the cell pellet resuspended in half its original volume with a chilled solution of 100mM $CaCl_2$, 70mM $MgCl_2$ and 40mM unbuffered sodium acetate. This was placed on ice for 30min and the cells centrifuged again before resuspending in 1ml of the above solution. These cells were now 'competent' for transformation and were stored at 4°C prior to use.

Transformation was carried out by adding 1μl of ligation mix (approximately 15ng DNA) to 100μl competent cells in a chilled tube (Falcon 2059, Beckton Dickinson & Co., Plymouth, England) and placed on ice for 30min. Plasmid was introduced into the cells by heat shock at 42°C for 45s, cells were returned to ice for 2min and then resuspended in 900μl SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose). Cells were incubated with vigorous shaking (225 rpm) for 1h at 37°C before 100μl of the cell suspension was spread onto LB-agar plates (appendix I) containing 50μg/ml ampicillin, 0.5M IPTG and 50μg/ml X-gal (LAXI plates) using a bent glass rod. Plates were inverted and incubated at 37°C overnight until bacterial colonies appeared. Identification of colonies transformed with plasmid containing the cDNA insert was based on the *lac Z* blue/white selection (see Fig.1a). The Stratagene Bluescript SK^- vector contains the *lac Z* gene in its polylinker region. *Lac Z* expression can be induced by IPTG to give its product ß–galactosidase. This can act on its substrate X-gal, the metabolism of which by ß–galactosidase results in a blue coloured end product. The *lac Z* gene is disrupted by the insertion of a cDNA sequence into the vector by ligation. Therefore bacterial colonies containing plasmid without the insert have a functional *lac Z* gene and are blue but those colonies which were successfully transformed with recombinant plasmid are white. White colonies were picked, restreaked onto LAXI plates and grown for a further 18h at 37°C.

Liquid cultures of the positive (white) colonies were grown overnight in LB-broth containing ampicillin and plasmid DNA was isolated using the Promega 'Magic Miniprep' kit (see section 3.5). To confirm that the purified plasmid DNA did contain the correct cDNA insert the plasmids were sequenced by chain termination using Sequenase (see section 3.11).

TA vector into INVαF' cells. E.coli cells (INVαF') were provided by InVitrogen already competent for transformation. The selection process for cells transformed with insert containing plasmids was essentially as for Bluescript (see Fig.1b). However, the plasmid lacks the *lac Z* repressor element and therefore does not need IPTG for induction of expression. Cells were transformed with 1μl of the ligation

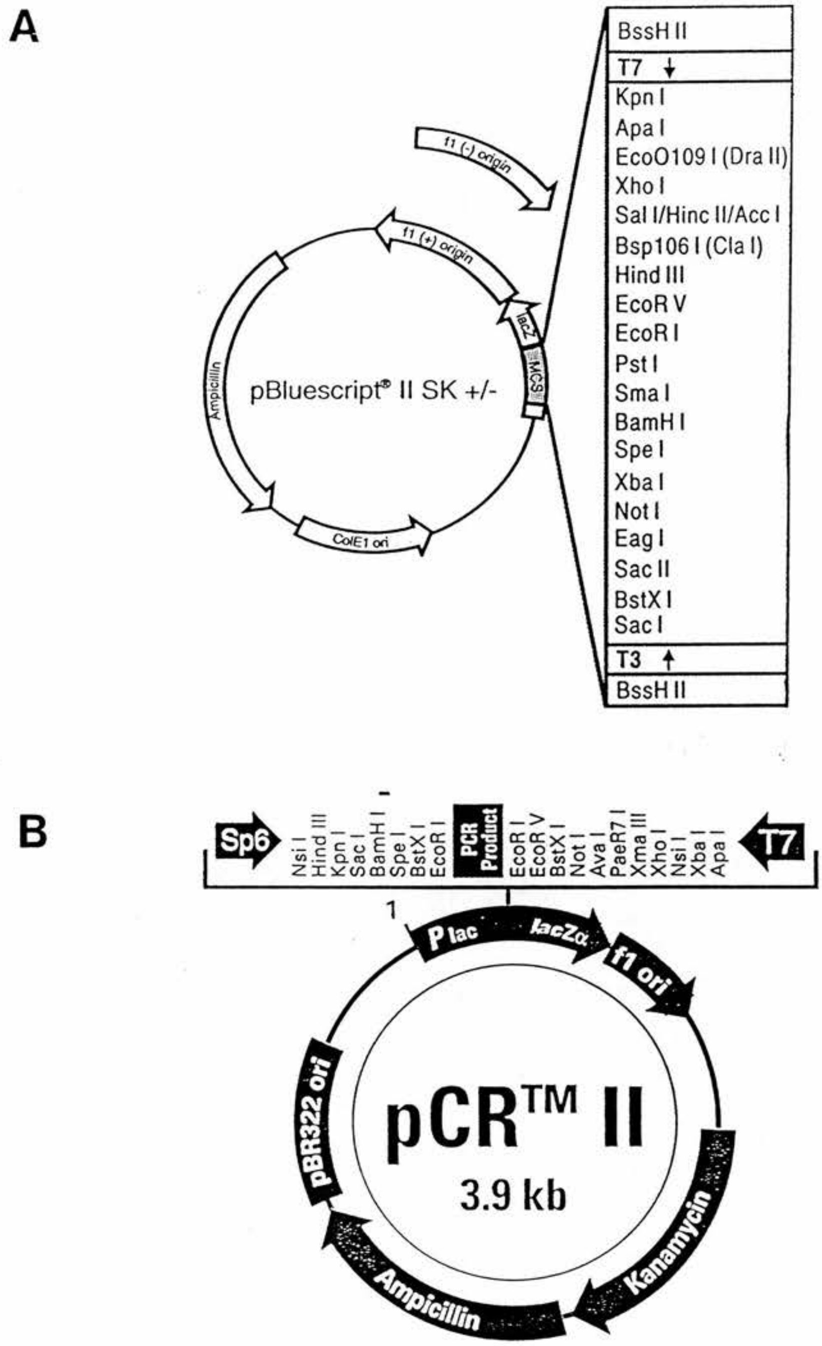


Figure 1. Maps of Bluescript SK- and TA cloning vectors. (A). Diagram of the Bluescript SK- cloning vector showing the polylinker region, sites for RNA polymerase binding (T3 and T7) and the genes for ampicillin resistance and ß-galactosidase which are the basis for selection of recombinants. *Adapted from Stratagene catalogue.* (B). Diagram of the pCR^{TM} II cloning vector showing the polylinker region, sites for RNA polymerase binding (SP6 and T7) and the genes for ampicillin resistance and ß-galactosidase which are the basis for selection of recombinants. *Adapted from InVitrogen catalogue.*

reaction without further purification using the heat shock method as described above. LB-agar plates containing 50µg/ml ampicillin and spread with 25µl X-gal (40mg/ml stock) were prepared (LAX plates). Transformed cells (25µl of suspension) were spread onto the plates and incubated inverted at 37°C overnight. White colonies were picked, restreaked onto LAX plates and incubated overnight at 37°C. Single colonies were cultured in LB-broth, plasmid DNA was prepared and the insert was sequenced to confirm its identity.

4.2.4 *In situ* hybridisation

Plasmids containing cDNA inserts were linearised to provide templates for transcription of riboprobes as described in chapter 3. The enzymes used to linearise the plasmids, the RNA polymerases which catalysed riboprobe formation and the orientation of the insert are shown in Table 1 for each of the Sertoli cell mRNAs studied.

Table 1. Details of conditions used to generate riboprobes.

Probe	Plasmid vector	Cutting enzyme	RNA polymerase	Orientation of insert
ABP	pSP6/T3	Hind III	T3	Antisense
"	"	Bgl II	SP6	Sense
α-inhibin	Bluescript	Bam HI	T7	Antisense
"	"	Hind III	T3	Sense
CRBP	pGem 3	Bam HI	T7	Sense
"	"	Pvu II	SP6	Antisense
CP-2	Bluescript	Bam HI	T7	Antisense
"	"	Hind III	T3	Sense
Cystatin C	Bluescript	Bam HI	T7	Antisense
"	"	Hind III	T3	Sense
SGP-1	pCR™ II	Kpn I	T7	Sense
"	"	Xho I	SP6	Antisense
SGP-2	pCR™ II	Kpn I	T7	Antisense
"	"	Xho I	SP6	Sense
Transferrin	SP64	Pst I	SP6	Antisense

Restriction enzymes used for digestion of plasmids and RNA polymerases used for transcription of riboprobes. Orientation of the cDNA inserts also shown.

Pretreatment of testicular tissue was as previously described in general methods (chapter 3). Hybridisation of probe to mRNA *in situ* was performed at 50°C with buffer containing 50% formamide for ^{35}S-UTP labelled probes and 37% formamide for DIG-labelled probes. Post-hybridisation washes were again essentially as described in chapter 3. Tissue hybridised with radiolabelled or DIG-labelled riboprobes were washed to 0.5 x SSC at room temperature and 0.1 x SSC/30% formamide at 40°C for 15min, respectively.

4.2.5 Immunohistochemistry

Immunostaining for cystatin C was performed as described in chapter 3. Rabbit antibody directed against human cystatin C (Cejka & Fleischman, 1973) was obtained from Dakopatts (code no. A 451). This antibody cross-reacted strongly with rat cystatin C and was used at a dilution of 1:50 to 1:150 in TBS.

4.3. Results

4.3.1 Localisation of transcripts

In situ hybridisation was performed to localise messenger RNAs to specific cell types in the testis. The use of ^{35}S-UTP labelled riboprobes for SGP-1, SGP-2, CP-2 and ABP showed a distinctive localisation of the transcripts around the base of the seminiferous epithelium (Fig.2). Digoxigenin labelled riboprobes were used to confirm cellular localisation of transcripts and it was shown that these were expressed specifically in the cytoplasm of Sertoli cells (Fig.3). Unequivocal identification of the cell type/s expressing CRBP or transferrin mRNA was not possible from the radioactive *in situ* hybridisation studies performed and localisation with DIG-labelled riboprobes was unsuccessful. The distribution of grains observed with these riboprobes was more diffuse and could have been attributed to expression in several cell types (Fig.4). Expression of mRNA for cystatin C using a radiolabelled riboprobe appeared to be in the germ cells of the seminiferous epithelium, specifically pachytene and diplotene spermatocytes, round spermatids and elongate spermatids at certain stages of the spermatogenic cycle (Fig.5a). The possible expression of cystatin C mRNA in Sertoli cells could not be ruled out from the results of *in situ* hybridisation on normal rat testis. However, hybridisation to sections from a Sertoli cell only rat testis showed that cystatin C mRNA was expressed in Sertoli cells (Fig.5b). This section also showed expression of the mRNA in cells of the interstitial space which were identified as macrophages.

4.3.2 Stage specific expression

Expression of all the mRNAs studied, with the exception of SGP-2, was shown to change according to the stage of the spermatogenic cycle. The pattern of expression of each transcript is shown in Table 2. ABP mRNA was highest at stages VII-XII of the cycle, began to decrease at stage XIII and there was no obvious expression at any other stage (see Fig.2). CP-2 mRNA was expressed only at stages IV-VII of the cycle. The level of CRBP mRNA was highest at stages VIII-XIII with no expression above background levels at stages XIV-VII (see Fig.4). Cystatin C mRNA was expressed heavily at all stages of the cycle with the exception of stage VIII where only a low level of signal was detected (see Fig.5). SGP-1 was expressed at all stages of the cycle but the highest levels were seen at stages XIV-VII. Expression of SGP-2 did not appear to change according to the stage of the spermatogenic cycle; it was observed to be heavily expressed in all Sertoli cells. Finally, transferrin mRNA was detected at the highest levels in stages XIV-VII, was low at stages VIII to XI and then began to increase again (see Fig.4).

4.3.3 Northern blot analysis

Identification of mRNA in the testis hybridising to selected ^{32}P-dCTP labelled cDNA probes was studied using Northern blot analysis as described in chapter 3.

α-inhibin. A 1.6kb transcript for α-inhibin was detected in the testis. This transcript was also found to be expressed in ovarian tissue but to a much greater extent than in testis (Fig.6, see lanes 1, 2 and 15).

ABP. A cDNA probe specific for ABP was found to hybridise to a single transcript in the testis of 1.7kb as shown by Hall *et al.* (1990). The hybridisation signal obtained was higher with RNA isolated from testes of 22 day old rats compared to the adult (Fig.7, compare lane 2 with lanes 1 and 15).

CP-2. Northern blot analysis of testicular RNA with a partial cDNA clone of CP-2 resulted in the detection of two mRNA transcripts of 1.7kb and 2.2kb. This was in agreement with data reported previously by Erickson-Lawrence *et al.* (1991). The 1.7kb transcript was significantly more abundant than the 2.2kb mRNA in the testicular samples while the 2.2kb transcript was more heavily expressed in total RNA prepared from kidney (data shown in chapter 6).

CRBP. A single transcript of approximately 0.7kb was detected with a CRBP specific cDNA probe. The signal was quite diffuse, perhaps indicating the existence of several differentially polyadenylated messages. RNA isolated from the epididymis was also shown to express this message at relatively high levels (data not shown).

Cystatin C. Two transcripts were detected on hybridisation of a cystatin C specific probe to total testis RNA (Fig.8, lane 1). These mRNAs were approximately 700 and 500bps as previously reported by Cole *et al.* (1989). The smaller transcript was found to be made up of two transcripts of approximately 500 and 550bps and these were expressed specifically in isolated fractions of germ cells (Fig. 9, lanes 1-6). Technique for isolation of germ cell fractions was as outlined in chapter 5, section 2.4.

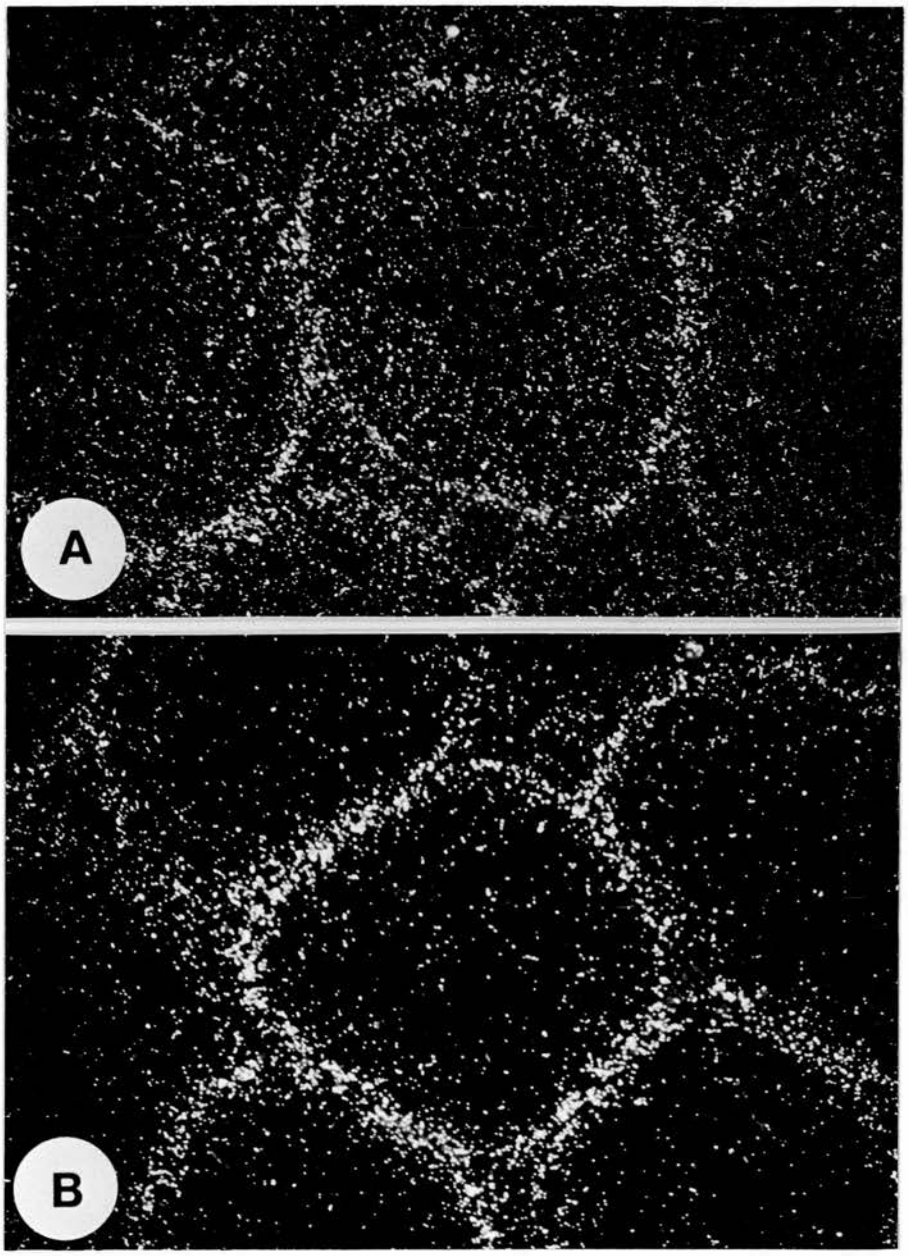


Figure 2. Cellular localisation of ABP and SGP-1 mRNA. Darkfield photomicrographs of the localisation and stage specific expression of (A) ABP and (B) SGP-1 on control rat testis using antisense ^{35}S-UTP labelled riboprobes. x90 magnification.

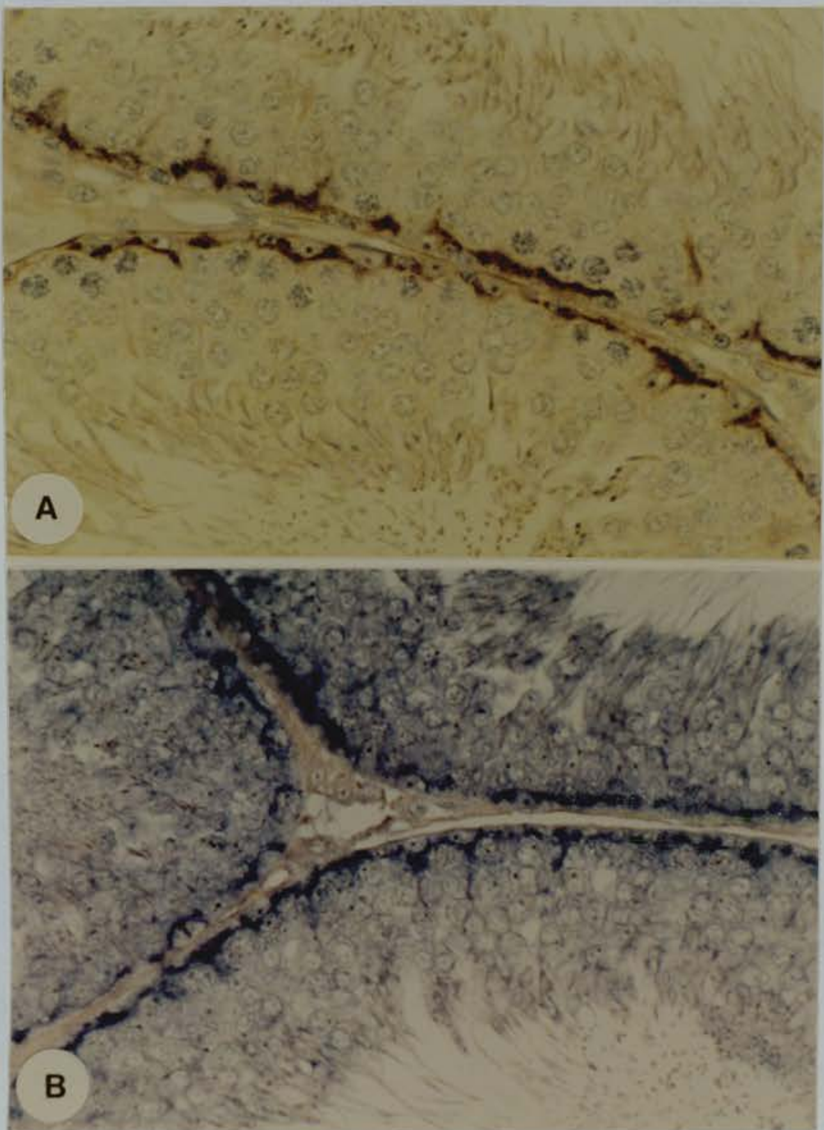


Figure 3. Sertoli cell localisation of CP-2 and SGP-2 mRNA. Brightfield photomicrographs showing localisation of (A) CP-2 and (B) SGP-2 to Sertoli cells in control rat testis using antisense Dig-UTP labelled riboprobes. x227 magnification.

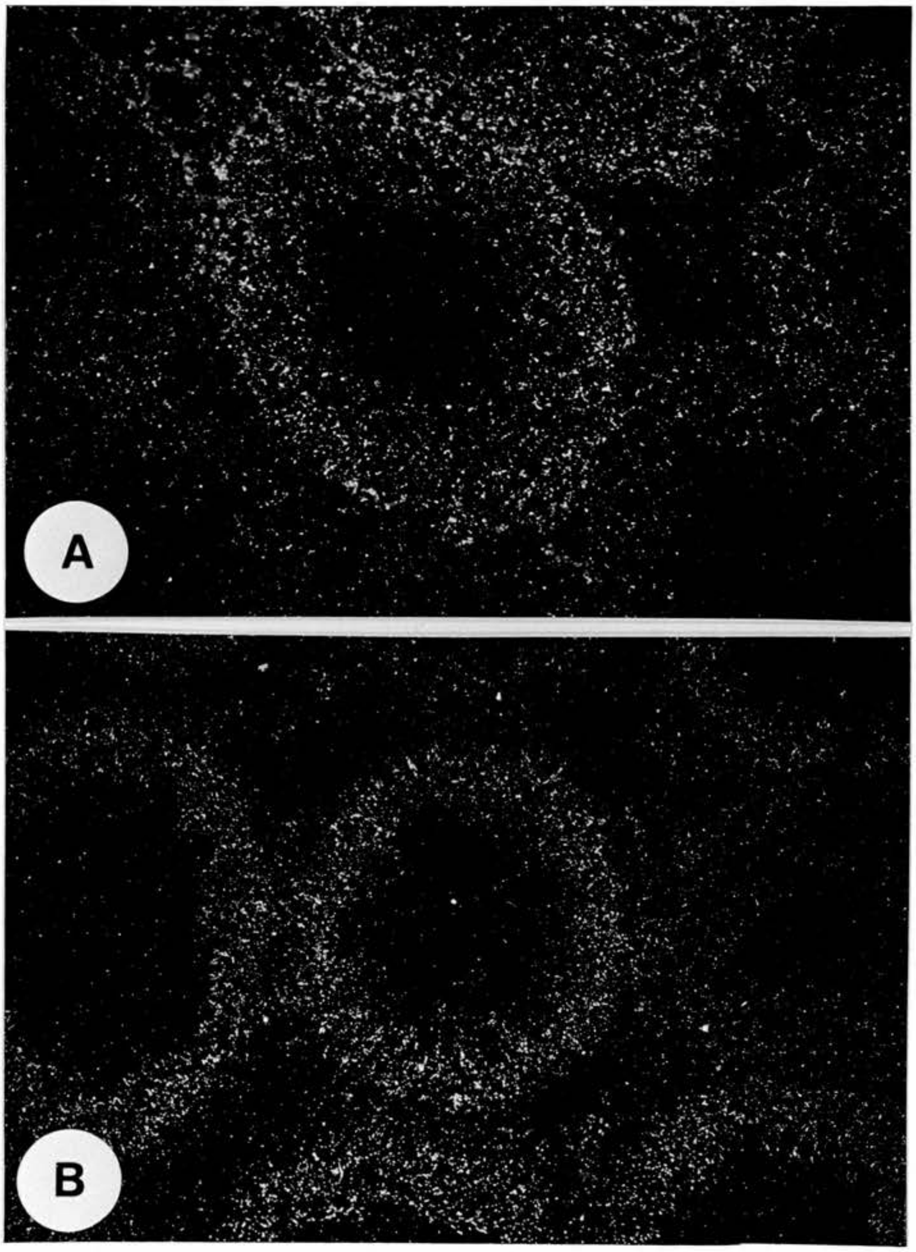


Figure 4. Cellular localisation of CRBP and transferrin mRNA. Darkfield photomicrographs of the localisation and stage specific expression of (A) CRBP and (B) transferrin on control rat testis using antisense ^{35}S-UTP labelled riboprobes. x90 magnification.

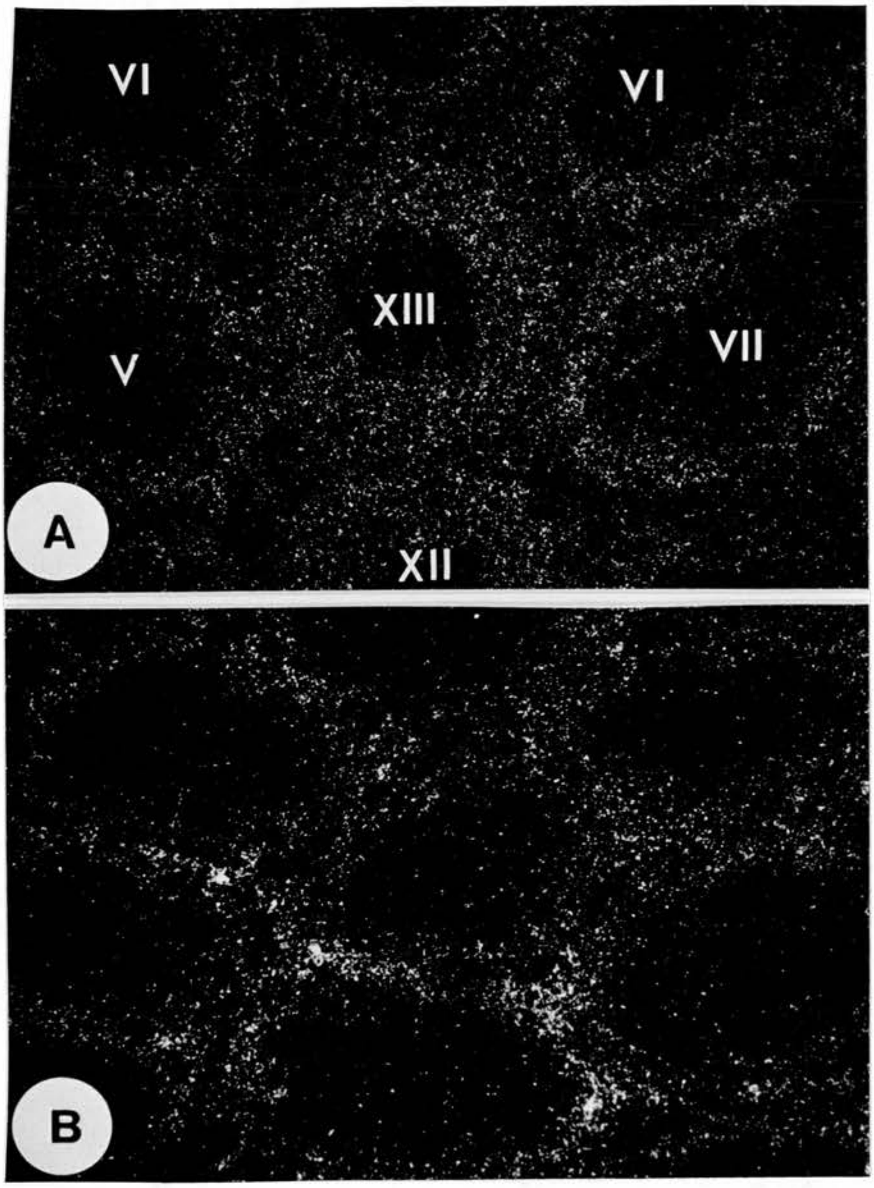


Figure 5. Expression of cystatin C mRNA. Darkfield photomicrographs of the localisation and stage specific expression of cystatin C on (A) control rat testis and (B) Sertoli cell only rat testis using antisense ^{35}S-UTP labelled riboprobes. x90 magnification.

Table 2. Stage specific expression of Sertoli cell mRNAs.

mRNA	Stage of the spermatogenic cycle													
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**	**XIII**	**XIV**
ABP	-	-	-	-	-	-	++	++	++	++	++	++	+	-
CP-2	-	-	-	++	++	++	++	-	-	-	-	-	-	-
CRBP	-	-	-	-	-	-	-	++	++	++	++	++	++	-
Cys C	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++
SGP-1	++	++	++	++	++	++	++	+	+	+	+	+	+	++
SGP-2	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Tf	+++	++	++	++	++	++	++	+	+	+	+	+	++	+++

Level of expression of the testis mRNAs studied at the fourteen different stages of the spermatogenic cycle. - = no. expression above background levels, + = low level of expression, ++ = medium expression and +++ = high level of expression. Assignment of the levels of expression was arbitrary and is for comparison within the one experiment. Comparison of the level of expression between the transcripts was not possible with the techniques used.

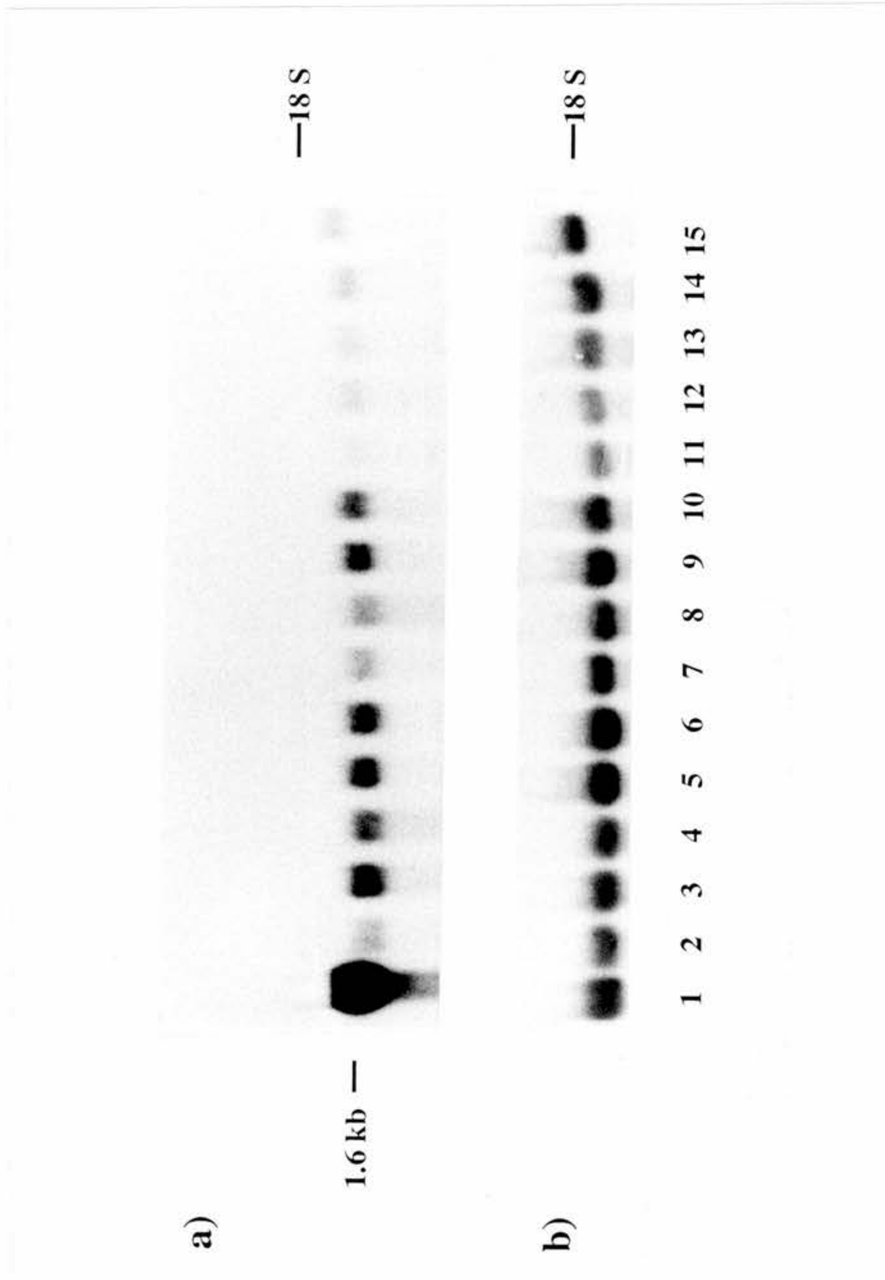


Figure 6. Northern blot analysis of α-inhibin mRNA expression. RNA samples are from control rat testis (lanes 2 and 15) and ovary (lane 1) and testes from each of two animals administered a single oral dose of methoxyacetic acid 3, 7, 14, 21, 28 or 42 days previously (lanes 3-4, 5-6, 7-8, 9-10, 11-12 and 13-14, respectively). All lanes were loaded with 15μg total RNA. The membrane was hybridised with ^{32}P-labelled α-inhibin cDNA and exposed to X-Omat AR film for 48 hours (A). The membrane was stripped and reprobed with an oligonucleotide probe for the 18S ribosomal RNA to check that all lanes contained equivalent amounts of RNA (B).

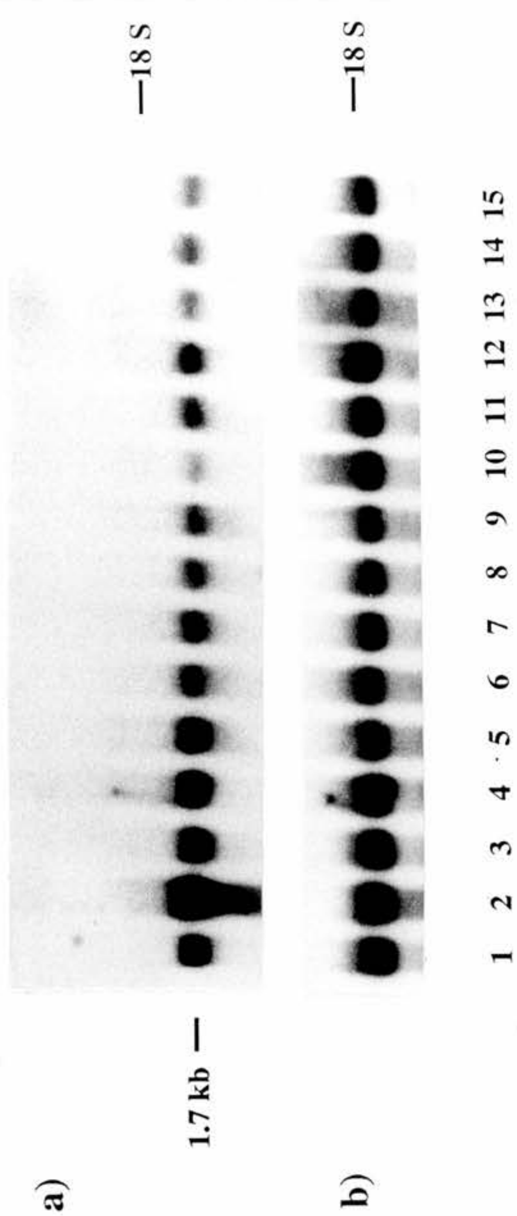


Figure 7. Northern blot analysis of ABP mRNA expression. RNA samples are from control adult rat testis (lanes 1 and 15), testis from 22d old male rat (lane 2) and testes from each of two adult animals administered a single oral dose of methoxyacetic acid 3, 7, 14, 21, 28 or 42 days previously (lanes 3-4, 5-6, 7-8, 9-10, 11-12 and 13-14, respectively). All lanes were loaded with 15μg total RNA. The membrane was hybridised with ^{32}P-labelled ABP cDNA and exposed to X-Omat AR film for 72 hours (A). The membrane was stripped and reprobed with an oligonucleotide probe for the 18S ribosomal RNA to check that all lanes contained equivalent amounts of RNA (B).

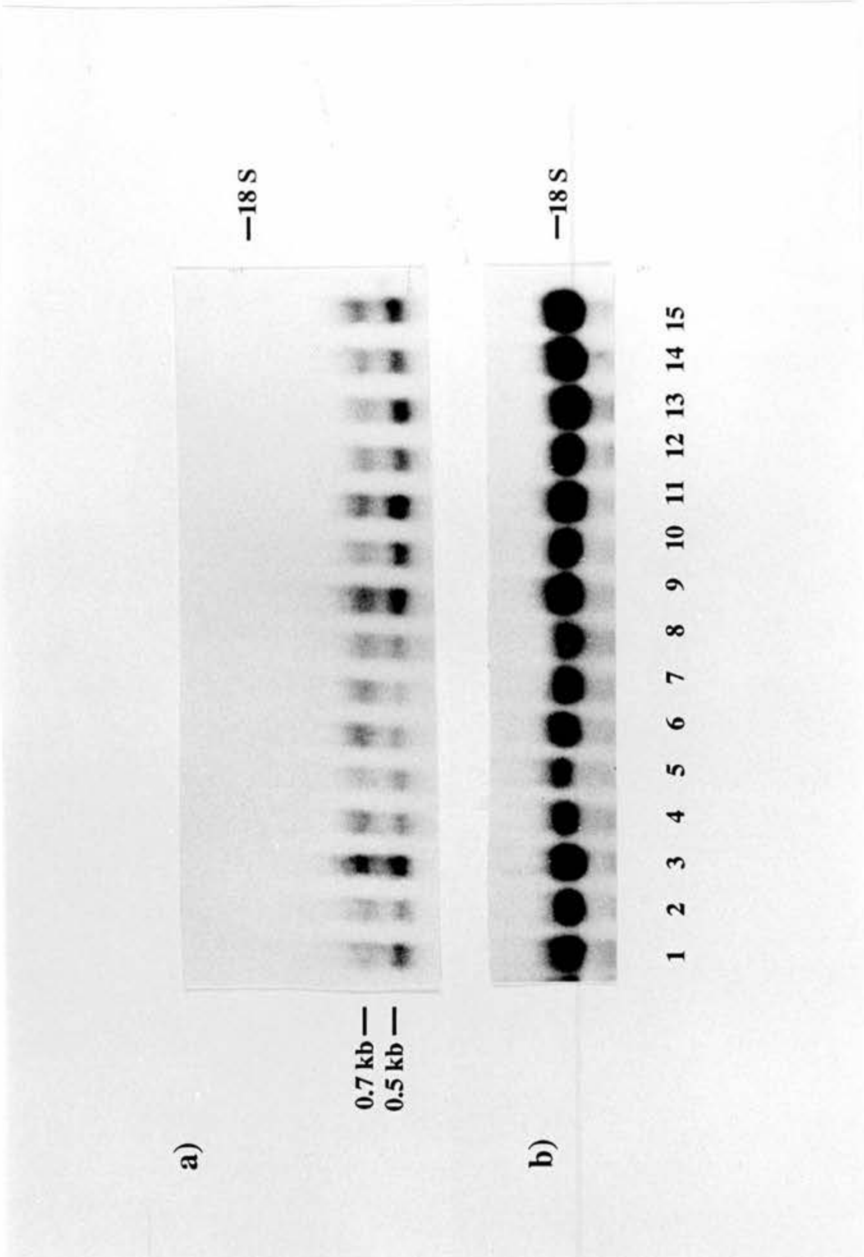


Figure 8. Northern blot analysis of cystatin C mRNA expression in germ cell depleted testis. RNA samples in panels A and B are from control rat testis (lanes 1 and 15), kidney (lane 2) and testes from each of two animals administered a single oral dose of methoxyacetic acid 3, 7, 14, 21, 28 or 42 days previously (lanes 3-4, 5-6, 7-8, 9-10, 11-12 and 13-14, respectively). All lanes were loaded with 15μg total RNA. The membranes were hybridised with ^{32}P-labelled cystatin C cDNA and exposed to X-Omat AR film for 24 hours (A). Membrane in A was stripped and reprobed with an oligonucleotide probe for the 18S ribosomal RNA to check that all lanes contained equivalent amounts of RNA (B).

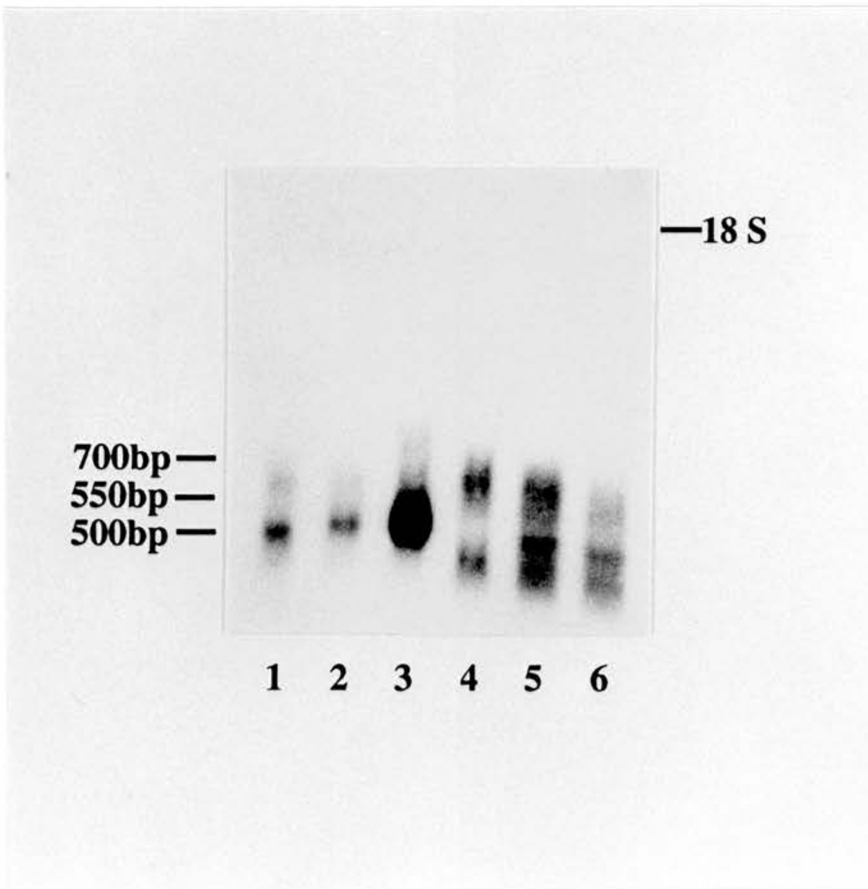


Figure 9. Northern blot analysis of cystatin C mRNA expression in germ cell enriched fractions. RNA samples are from isolated cell fractions containing enriched preparations of spermatogonia and early spermatocytes (lane 1), round spermatids (lanes 2 and 6), elongate spermatids (lane 3) and pachytene spermatocytes (lanes 4 and 5). Lanes 1-4 contain 15μg of total RNA and lanes 5 and 6 contain 2μg of mRNA.The membrane was hybridised with ^{32}P-labelled cystatin C cDNA and exposed to X-Omat AR film for 24 hours.

SGP-1. Analysis of total testis RNA showed the presence of a single transcript of approximately 2.6kb specific for SGP-1, as originally shown by Collard *et al.* (1988). This transcript was also present in RNA isolated from kidney (not shown).

SGP-2. A probe specific for SGP-2 mRNA was found to hybridise to a transcript of approximately 2.0kb in the testis, as shown by Collard & Griswold (1987). RNA isolated from rat kidney tissue did not hybridise to the probe (not shown).

Transferrin. The probe used to detect transferrin in the testis identified two transcripts, one of 2.6kb and a more abundant message of 0.9kb (see Stallard *et al.*, 1991). Hybridisation of the cDNA to ovarian tissue only detected the 2.6kb mRNA (data shown in chapter 5).

4.3.4 Identification of possible germ cell regulation of transcripts

Northern blot analysis. MAA treatment of adult rats was used to deplete the testis of specific germ cell types. Northern blot analysis with the probes described above was performed as an initial screen to detect any major effects of germ cell depletion from the testis on expression of these mRNAs.

Table 3. Effect of germ cell depletion on the level of specific mRNAs.

Probe	**Changes in specific mRNA transcript/s with MAA treatment**
α-inhibin	small increase at 3d, 7d and 21d
ABP	no consistent changes
CP-2	small increase at 7d and 14d, decrease at 21d
CRBP	none
Cystatin C	small decrease in LMW transcript at 7d and 14d
SGP-1	none
SGP-2	none
Transferrin	LMW transcript decrease at 7d and 14d

Changes observed with germ cell depletion in signal obtained on autoradiographs of Northern blots probed with ^{32}P-dCTP labelled cDNAs. Transcript levels were determined by densitometric scanning. LMW=low molecular weight transcript, HMW=high molecular weight transcript.

The autoradiographs obtained were scanned densitometrically to detect any subtle changes in level of expression. An oligonucleotide probe for 18S rRNA was used to check for equal gel loading and membrane transfer (see chapter 3), and the ratio of specific hybridisation: 18S rRNA signal on the autoradiograph was calculated to give a more accurate reflection of changes in expression. Table 3 gives a summary of results obtained by Northern blot analysis. A typical result obtained for α-inhibin is shown in Fig.10.

In situ hybridisation. The effect of germ cell depletion on Sertoli cell mRNAs was studied in more detail using *in situ* hybridisation. This enabled identification of any stage specific changes in mRNA expression which could not be seen by Northern blot analysis where total testicular RNA had been isolated. *In situ* hybridisation confirmed that there was no major effect on pattern of transcription of mRNAs for ABP, CRBP, SGP-1 or SGP-2 when either pachytene spermatocytes, round spermatids or elongate spermatids were depleted from the seminiferous tubules (see Fig.11 for selected data on ABP, CRBP and SGP-1; data not shown for SGP-2). *In situ* hybridisation with a riboprobe directed against α-inhibin was not successful, so the change observed with Northern blot could not be confirmed in this way.

Expression of mRNA for cystatin C was reduced in specific tubules at all time points after MAA treatment analysed (Fig.12). This was due to the loss of signal associated with the germ cells (see Fig.9) although a change in expression (either increase or decrease) of cystatin C mRNA in Sertoli cells could not be ruled out.

The frequency of tubules expressing CP-2 mRNA was seen to be significantly reduced 21 days after MAA treatment compared to controls (Fig.13). At this time point it is mainly elongate spermatids which are depleted from the tubules. Transferrin mRNA expression was decreased in tubules at 7 days and 14 days after MAA treatment. This decrease was obvious only in tubules which had been depleted of round spermatids (Fig.14).

4.3.5 Immunolocalisation of cystatin C

Antibody directed specifically against cystatin C localised the protein to the Sertoli cells of the seminiferous epithelium (Fig.15a). Protein also appeared to be present in elongate spermatids but was not detected in any other germ cell type. Cystatin C was detected in Sertoli cells of animals at all days after MAA treatment (not shown) and in Sertoli cell only testis (Fig.15b). Specificity for cystatin C was confirmed as no staining was apparent when normal rabbit serum was used as the primary antibody (Fig.15c).

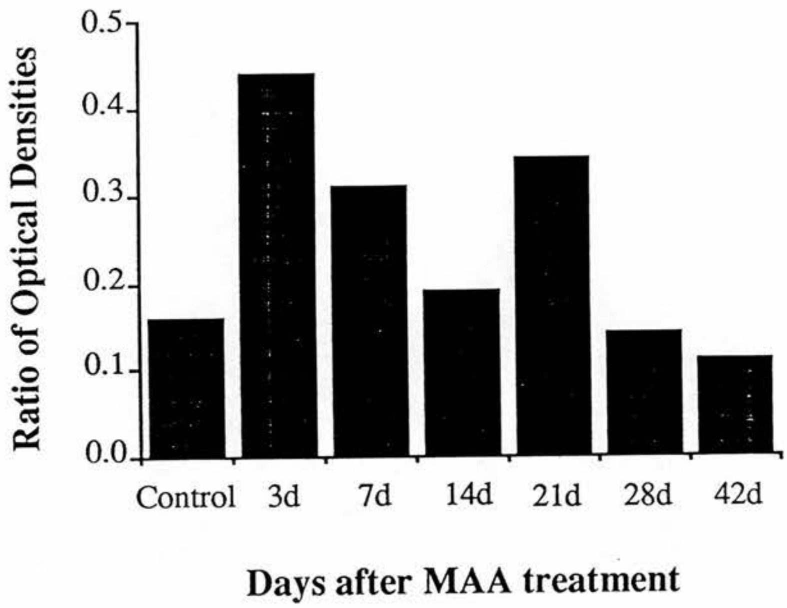
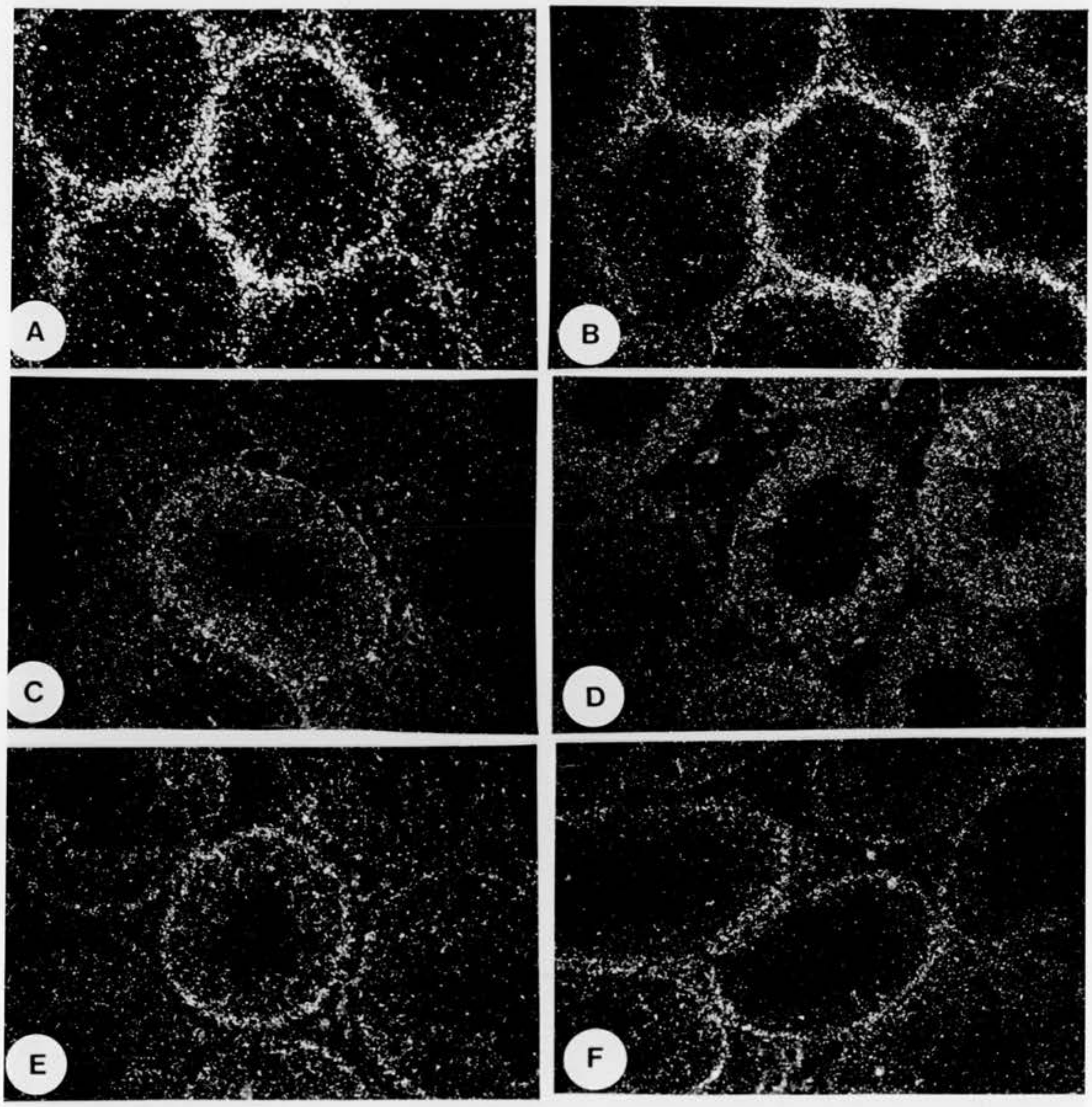


Figure 10. Effect of germ cell depletion on α-inhibin mRNA expression. Optical density of signals from panels A and B from Fig.6 measured using image analysis.The ratios of α-inhibin mRNA expression to 18S rRNA loading are plotted for each MAA time point to show the effect of treatment. C, control testis; 3, 7, 14, 21, 28 & 42 days after MAA treatment.

Figure 11. Effect of germ cell depletion on SGP-1, CRBP and ABP mRNA expression. Darkfield photomicrographs illustrating the lack of effect of germ cell depletion on expression of the Sertoli cell mRNAs SGP-1 (A, B), CRBP (C, D) and ABP (E, F). Sections are of control rat testis (A, C, E), and testis 3d (B), 14d (D) and 21d (F) after MAA treatment when pachytene spermatocytes, round spermatids or elongate spermatids, respectively, are depleted from the seminiferous tubules. x52 magnification.


A
B
C
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E
F

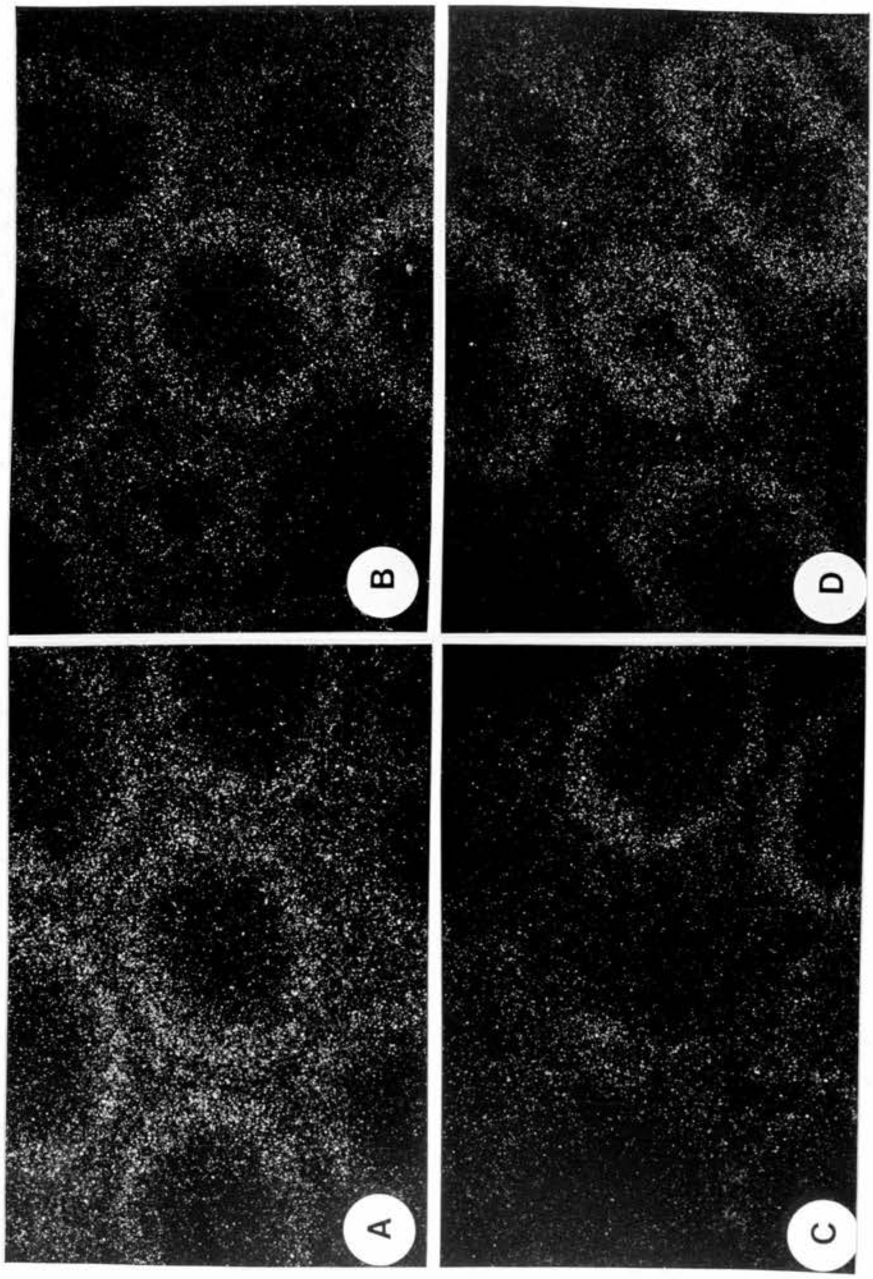


Figure 12. Effect of germ cell depletion on cystatin C mRNA expression. Darkfield photomicrographs illustrating the effect of germ cell depletion on expression of cystatin C mRNA. ^{35}S-UTP labelled antisense riboprobe was hybridised to control rat testis (A), and testis 3d (B), 14d (C) and 21d (D) after MAA treatment when pachytene spermatocytes, round spermatids or elongate spermatids, respectively, are depleted from the seminiferous tubules. x52 magnification.

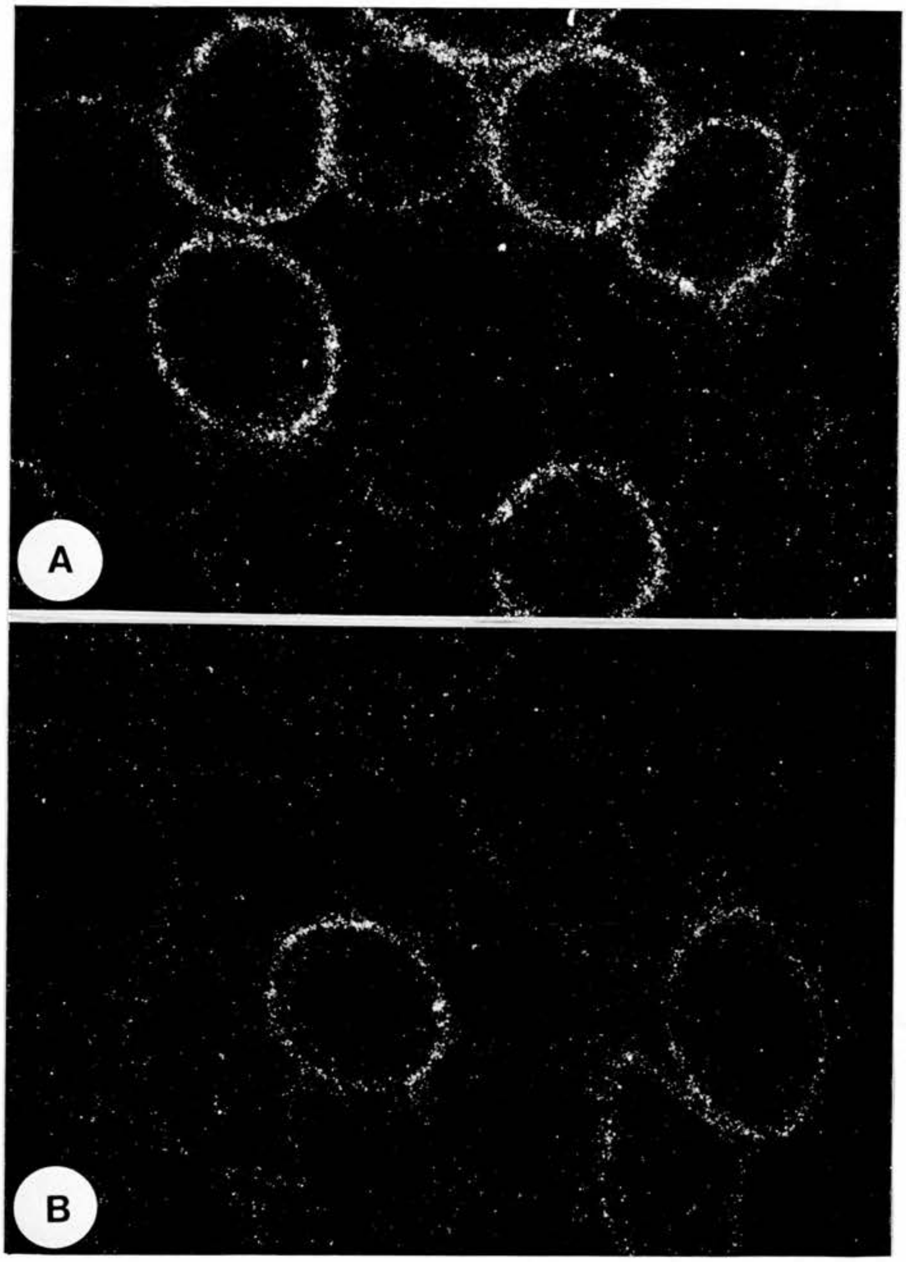


Figure 13. Effect of germ cell depletion on CP-2 mRNA expression. Darkfield photomicrographs illustrating the effect of depletion of elongate spermatids on expression of CP-2 mRNA. ^{35}S-UTP labelled riboprobe was hybridised to control rat testis (A) and testis 21d (B) after MAA treatment when elongate spermatids are depleted from the seminiferous tubules. x90 magnification.

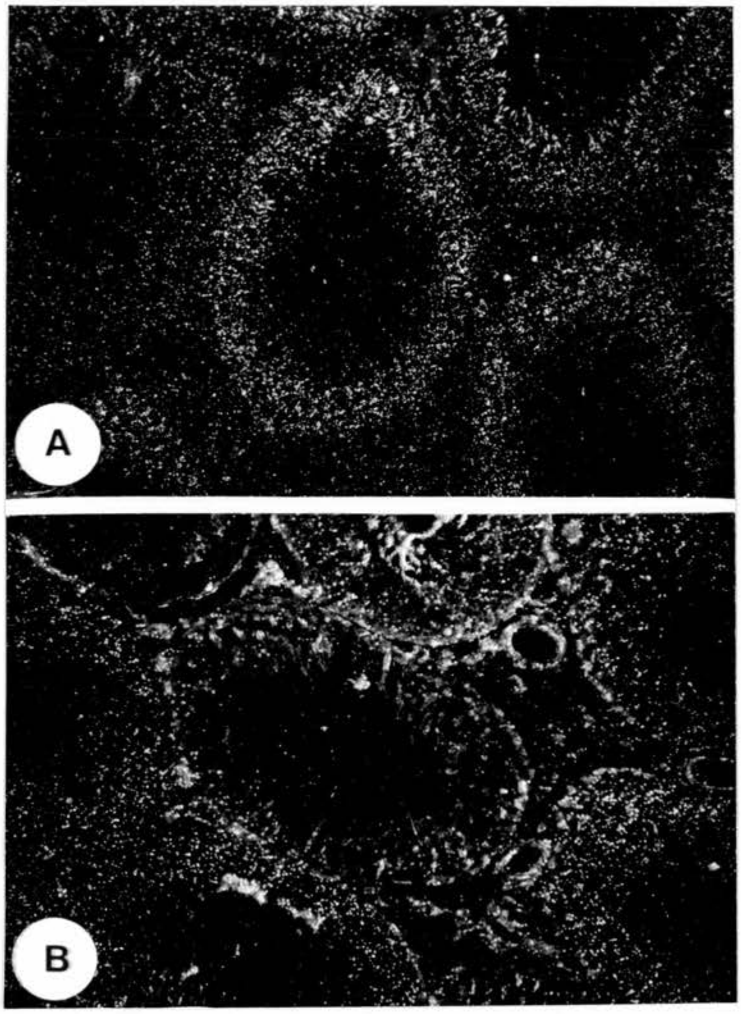


Figure 14. Effect of germ cell depletion on transferrin mRNA expression. Darkfield photomicrographs illustrating the effect of depletion of round spermatids on expression of transferrin mRNA. ^{35}S-UTP labelled riboprobe was hybridised to control rat testis (A) and testis 7d (B) after MAA treatment when round spermatids are depleted from the seminiferoustubules. x90 magnification.

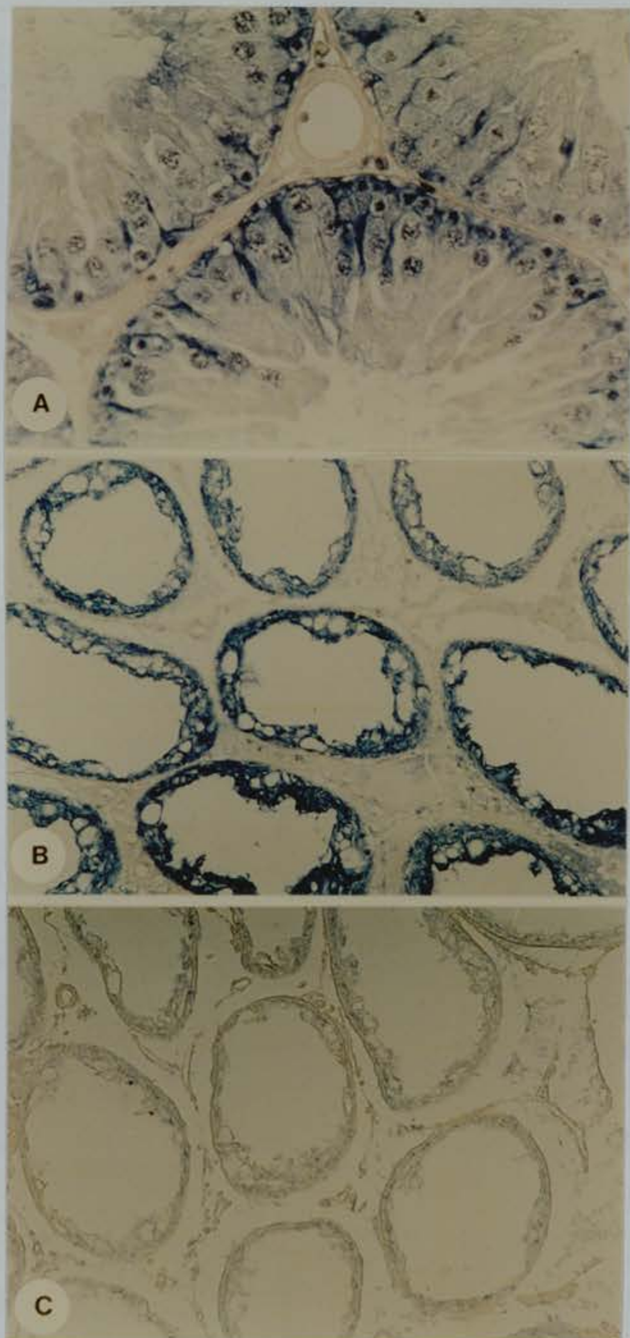


Figure 15. Localisation of cystatin C. Brightfield photomicrographs showing immunohistochemical localisation of cystatin C protein in the testis. Cross sections of control rat testis (A) and Sertoli cell only testis (B) incubated with an anti-human cystatin C which cross reacts strongly with the rat protein. Control rat testis incubated with normal rabbit serum as primary antibody used as a negative control is also shown (C). (A) x129 magnification, (B) & (C) x52 magnification.

4.4. Discussion

The influence of germ cells on Sertoli cell function is now well accepted. Numerous studies have shown that the addition of germ cells to Sertoli cells in culture can effect the secretion of specific proteins. However the majority of these studies have been performed using Sertoli cells isolated from immature animals (usually 18-25 day old rats). The first wave of spermatogenesis has not been completed in these rats and the most advanced germ cell type present within their seminiferous epithelium is usually pachytene spermatocytes (Clermont & Perry, 1957). Therefore these studies involve the addition of germ cell types isolated from mature rat testis to Sertoli cells which *in vivo* would not have been exposed to them. The function of the Sertoli cell changes as it matures, either as a consequence of exposure to the developing germ cells or as an integral function of the Sertoli cell itself (Waites *et al.*, 1985). Therefore, the response of immature Sertoli cells to contact with mature germ cells may not be the same as occurs *in vivo* in the adult animal. The studies described in this chapter were designed using an *in vivo* model of germ cell depletion in the adult rat to avoid obtaining aberrant results due to the use of immature Sertoli cells.

The effect of the toxicant methoxyacetic acid (MAA) on the adult rat has been well characterised (Bartlett *et al.*, 1988; Allenby, 1990). At a dose of 650 mg/kg it appears to have no adverse affects on the animals well-being and its only noticeable effect is to produce stage specific depletion of selected germ cells. The precise mechanism of the harmful effect of MAA is unknown but it has been suggested that it may interfere in some way with energy metabolism (Williams & Foster, 1988). Prior to degeneration, mitochondrial swelling is observed in pachytene spermatocytes which may be consistent with an effect on energy metabolism (Foster *et al.*, 1987). It is also of interest to note that pachytene spermatocytes which are resistant to MAA, those at early to mid stage VII of the spermatogenic cycle, are found to contain the highest levels of cytochrome c oxidase II mRNA (involved in the electron transport system) which is transcribed from the mitochondrial genome (Saunders *et al.*, 1993). In spite of its exact method of action remaining unclear, MAA does provide a useful tool to study the influence of germ cells on Sertoli cell function in the adult rat.

Two methods were used to study germ cell regulation of mRNA expression in Sertoli cells after MAA treatment; Northern blot analysis and *in situ* hybridisation. The use of Northern blot hybridisation was useful in two ways. Firstly, it enabled confirmation that the probe prepared was specific for the Sertoli cell product being studied as the hybridisation profile obtained could be compared to the published data. The approximate size of and the number of transcripts could be determined to check that the probe did not cross hybridise to other mRNAs. This was found to be important in the cases of transferrin and cystatin C as discussed later. Secondly, Northern blots were useful for screening for potential germ cell regulated mRNAs. This would only detect gross changes in the level of expression of transcripts but was useful as an initial approach to the study. The second method applied was *in situ* hybridisation, which proved useful in three ways. Firstly, it enabled cellular localisation of the messenger RNAs. Radioactive *in situ* hybridisation gave a good indication as to the localisation of the messages but precise identification of the cell type expressing the mRNA was only possible using digoxigenin-labelled riboprobes. Secondly, visualisation of riboprobe hybridisation *in situ* enabled the identification of specific stages of the spermatogenic cycle at which the mRNAs were expressed. Finally, a much more detailed study of the influence of particular germ cells on stage dependent Sertoli cell mRNA expression was possible with *in situ* hybridisation as compared to that obtained with Northern blots alone.

These studies showed the expression of ABP mRNA occurs in a cyclical manner with maximal expression at stages VII-XII of the spermatogenic cycle. A similar distribution of ABP mRNA has been shown in the testis of stage synchronised rats (Linder *et al.*, 1991). These are the same stages at which the highest levels of ABP secretion have been observed (Parvinen, 1982). This pattern of secretion may be significant since stages VII-VIII of the cycle are known to be the most dependent on testosterone for the progression of germ cells through the spermatogenic cycle (see Kerr *et al.*, 1993). ABP has been proposed to play a role in the influence of testosterone on the control of spermatogenesis but its exact function is unknown. Depletion of germ cells from the seminiferous epithelium did not appear to have any effect on the level of expression of ABP mRNA in the Sertoli cell as visualised by either Northern blot or *in situ* hybridisation. However, previous reports have indicated that germ cells are involved in the regulation of ABP production. The addition of pachytene spermatocytes to Sertoli cells *in vitro* stimulated the secretion of ABP (Galdieri *et al.*, 1984). These Sertoli cells were isolated from 25 day old rats and therefore a direct comparison with results obtained in the adult *in vivo* is not possible. However, *in vivo* studies in the adult rat have also been carried out and show an effect of germ cells on ABP production. Depletion of late spermatids from the seminiferous tubules achieved by maturation-depletion after either MAA treatment or γ-irradiation correlated with a decrease in the level of ABP secretion (Bartlett *et al.*, 1988; Pineau *et al.*, 1989). The absence of elongate spermatids did not appear to affect ABP mRNA levels in the studies described in this chapter. This may indicate that the stimulatory effect of elongate spermatids on Sertoli cell ABP production occurs at a post-transcriptional level either in control of the rate of translation of the mRNA, by altering post-translational modifications or the rate of secretion of the mature protein.

Previous studies have demonstrated that germ cells can influence inhibin production by Sertoli cells. A study using MAA depletion of germ cells showed that elongate spermatids appear to positively regulate inhibin secretion as indicated by a decrease in immunoreactive inhibin measured in plasma at 21 days after MAA treatment (Allenby *et al.*, 1991). Inhibin is known to inhibit the release of FSH from the pituitary gland and therefore circulating FSH levels are often used as an indirect measurement of inhibin production by Sertoli cells. FSH levels were shown to increase at a time after γ-irradiation when late spermatids were depleted from the seminiferous epithelium providing further evidence for a positive influence of late spermatids on inhibin secretion (Pineau *et al.*, 1989). The data generated in the present study showing an apparent increase in α-inhibin mRNA by Northern blot analysis when elongate spermatids are absent are inconsistent with these studies on inhibin protein secretion. However, the influence of germ cell depletion on the total amount of inhibin synthesised by Sertoli cells has not been determined; the studies reported above have measured changes only in the amount of inhibin secreted. Therefore the apparent difference between the influence of late spermatids on inhibin mRNA levels and its secretion may actually be real, with these germ cells decreasing total synthesis of inhibin mRNA but increasing its rate of translation and secretion. The amount of inhibin measured in interstitial fluid was not shown to be influenced by depletion of late spermatids suggesting that there was a decrease only in its apical secretion via seminiferous tubule (ST) fluid, which is the main route by which inhibin reaches peripheral blood in the adult rat (Allenby *et al.*, 1991). ST fluid produced by the Sertoli cell is important for the transfer of nutrients to the germ cells and for the transport of the mature spermatozoa to the epididymis (Jégou, 1992). The size of the lumen of the seminiferous tubules is thought to be influenced by the amount of ST fluid secreted and in turn lumen size has been shown to be positively regulated by elongate spermatids (Sharpe, 1989). The rate of secretion of factors produced by the Sertoli cell is obviously influenced by the production of ST fluid and therefore the decrease in inhibin secretion demonstrated on depletion of elongate spermatids may be a result of a decrease in ST fluid production. However, since stage-dependent measurement of ST fluid production is not yet possible this cannot be conclusively proved.

The current study also showed that depletion of pachytene spermatocytes caused an increase in the levels of α-inhibin mRNA. This confirms the finding of other studies in which Northern blot analysis of microdissected staged tubules from animals at specific times after γ-irradiation showed an increase in α-inhibin mRNA at stages VII and VIII of the cycle when either pachytene spermatocytes or round spermatids were depleted from the tubules (Kaipia *et al.*, 1991). However, serum FSH levels were not changed by depletion of pachytene spermatocytes indicating either that inhibin secretion was not affected or if it was increased that only immunoreactive and not bioactive inhibin was being secreted (Allenby *et al.*, 1991). The present study also showed an increase in inhibin mRNA at 7 days after MAA treatment when either pachytene spermatocytes or round spermatids were depleted from tubules at specific stages of the spermatogenic cycle. However, at 14 days after treatment when round or elongating spermatids were depleted there was no significant increase observed. This suggests that the increase observed at 7 days after MAA was due to depletion of pachytene spermatocytes from the tubules while depletion of round spermatids did not affect inhibin mRNA levels. The results could also have been attributable to a stage specific effect of round spermatids on Sertoli cell inhibin mRNA expression in Sertoli cells but this seems unlikely. At 7 days after MAA round spermatids are depleted from stages II-V of the spermatogenic cycle while stages I-VI are depleted of these cells at 14 days after treatment. Therefore, round spermatids are depleted from stages II-V at both time points after MAA but an increase in inhibin mRNA is only observed at 7 days when pachytene spermatocytes are also depleted from tubules at specific stages of the spermatogenic cycle. A previous *in vivo* study of immunoreactive inhibin secretion also failed to show an effect of depletion of round spermatids (Allenby *et al.*, 1991). The conclusion reached by analysis of the published studies and the present one is that although the exact influence of the different germ cells on inhibin mRNA expression and its secretion is confused, it seems likely that germ cells do have a role to play in regulating inhibin production by Sertoli cells. A successful *in situ* hybridisation study should help to resolve this.

The expression of CRBP mRNA by Sertoli cells was not influenced by the depletion of germ cells from the testis. The marked stage specificity of CRBP mRNA had suggested that germ cells may be involved in the regulation of its expression. However, this was not seen to be the case using the current experimental design although the possibility still remains that synthesis or secretion of the protein may be influenced by the presence of specific germ cells. The level of CRBP mRNA has been shown to be regulated by retinoids (Rajan *et al.*, 1990b). Animals fed a retinoid-deficient diet show reduced expression of CRBP mRNA but the stage specific expression is unaffected. The level of expression can be returned to normal by supplementation of the diet with retinol. Germ cells in the adluminal compartment of the seminiferous epithelium are found to contain cellular retinoic acid-binding protein (CRABP) but not CRBP (Porter *et al.*, 1985). Both germ cells and Sertoli cells express mRNA for retinoic acid receptor (Kim & Griswold, 1990). From these studies it has been proposed that retinol in plasma is bound to retinol binding protein (RBP) where it may be stored or converted to retinoic acid and transported to germ cells where it is uptaken by CRABP. Whilst the exact role of these retinoids in the testis is unclear, disruption of normal spermatogenesis in the absence of retinoids would be consistent with an important role in the process.

The marked stage specific secretion of CP-2 by the Sertoli cell, with maximal levels occurring at stages VI-VII, indicates that it may be modulated by germ cells. The stage specificity was confirmed at the mRNA level as shown previously (Erickson-Lawrence *et al.*, 1991). Expression of mRNA for CP-2 was influenced by germ cell depletion following MAA treatment. The main finding of this study was a decrease in expression 21 days after treatment when late spermatids were depleted from the tubules as observed both by Northern blot and *in situ* hybridisation. These results were the first report of a germ cell effect on Sertoli cell production of CP-2. Therefore, the influence of germ cells on CP-2 synthesis was studied in more detail. The results and discussion of these studies are presented in chapter 6.

The marked remodelling of the seminiferous epithelium which occurs throughout the spermatogenic cycle due to the movement of elongate spermatids indicates an important role for proteases in the testis (Fritz *et al.*, 1993). The recent discovery that CP-2, one of the most abundant Sertoli cell secreted proteins, is the proenzyme form of the cysteine protease cathepsin L is consistent with this idea (Erickson-Lawrence *et al.*, 1991). The presence of proteases in the testis in turn suggests that inhibitors of these proteases may also be expressed. However, to date there is little evidence for the presence and/or role of protease inhibitors in the testis. Cathepsin L has a ubiquitous tissue distribution (Chauhan *et al.*, 1991) and is known to be inhibited by members of the cystatin superfamily of cysteine proteinase inhibitors (Barrett, 1987). However, it is bound with the highest affinity by cystatin C and therefore it was of interest to study the distribution of this inhibitor in the testis. Secretion of this protein by Sertoli cells in culture had previously been shown (Esnard *et al.*, 1992) but no studies on its stage specific expression or production by other cell types in the testis had been undertaken. Results from the present study showed expression of cystatin C mRNA in Sertoli cells and all germ cells more mature than and including pachytene spermatocytes. However, the protein was only detected in Sertoli cells and elongate spermatids. This would suggest that control of mRNA translation differs in the individual cell types. However, another explanation for this discrepancy in mRNA and protein localisation may be found in the presence of cystatin C transcripts of different lengths in the testis. It is possible that only one of the mRNA transcripts detected is translated. A recent study by Tsuruta and co-workers (1993) has also suggested this possibility. This group detected a 700bp transcript in testis which was exclusively expressed in Sertoli cells while smaller transcripts of 550 and 500bps were expressed in round and elongating spermatids, and pachytene spermatocytes, respectively. The germ cell transcripts were found to be different only in the length of their poly A tails while the larger size of the Sertoli cell transcript was not due only to differential polyadenylation. The authors suggested that the 700bp Sertoli cell transcript is translated and the protein may be transferred to elongate spermatids. However, it is more likely that these germ cells synthesise their own cystatin C since they contain their own messenger RNA.

Cystatin C mRNA has been shown to be expressed in a stage dependent manner in Sertoli cells (Tsuruta *et al.*, 1993). Highest levels of the message were found at stages X-XIII and lowest levels at stages VI-VII of the spermatogenic cycle. This expression pattern is the mirror image of that seen for cathepsin L mRNA in the Sertoli cell, where expression is maximal at stages IV-VII (Erickson-Lawrence *et al.*, 1991). It has been proposed by Tsuruta and co-workers (1993) that this pattern of expression enables cystatin C to control the action of the protease cathepsin L in the seminiferous epithelium. However, it would appear that the Sertoli cell is already controlling the action of cathepsin L by regulating the stages of the cycle at which it is produced. Cystatin C mRNA levels are highest at the later stages of the cycle but there is no expression of CP-2 mRNA at these stages and nor has the protein been detected. This would suggest that it is not necessary to produce an inhibitor to limit the protease actions. However, low levels of expression of cystatin C at stages VI-VII when CP-2 is maximally produced are consistent with the protease having an important function at these stages. Although cystatin C inhibits cathepsin L with high affinity it is known to inhibit other proteases, such as cathepsin B and H (Barrett, 1987). The distribution of these proteases in the testis has not been investigated to my knowledge and it may be possible that cystatin C has a role in inhibiting proteases other than cathepsin L in the testis. Whatever the interaction of cathepsin L and cystatin C in the testis it is generally agreed that proteases and their inhibitors have important roles to play in the process of spermatogenesis. However, further studies are required to determine the exact sites and mechanism of their action.

Sertoli cell transferrin production also appeared to be regulated by germ cells as indicated by the stage specific mRNA expression and as shown directly by *in situ* hybridisation. The depletion of round spermatids from the seminiferous tubules caused a decrease in the level of transferrin mRNA. A stimulatory effect of round spermatids on synthesis of transferrin by Sertoli cells has previously been shown which is consistent with the present finding (Le Magueresse *et al.*, 1988). However, the present study showed that the cDNA probe used hybridised to two mRNA transcripts on Northern blot and it has to be assumed that both of these mRNAs are localised during *in situ* hybridisation studies. The smaller 0.9kb transcript detected has been reported in the literature as hemiferrin, a mRNA found to be expressed in germ cells, specifically round spermatids (Stallard *et al.*, 1991). This finding led to concern that the decrease in transferrin mRNA expression observed on depletion of round spermatids reported here may have been due to loss of the hemiferrin transcript and not to an influence of round spermatids on expression of transferrin mRNA by Sertoli cells. Therefore, it was necessary to engineer a probe which would hybridise only to the transcript corresponding to Sertoli cell transferrin. A full account of the results obtained with the original and the transferrin specific probe is contained in chapter 5.

SGP-1 mRNA was observed to be expressed in a stage specific manner in the seminiferous epithelium but SGP-2 appeared to be expressed at similar levels at all stages of the spermatogenic cycle. These results are in contrast to those obtained by Morales *et al.* (1989). These authors found the level of SGP-1 mRNA remained constant at all stages of the spermatogenic cycle while SGP-2 mRNA was stage specifically expressed, with the highest levels at stages VII and VIII. The difference in these results may be due to the methods used. The present study used normal adult rats and identification of the 14 stages of the spermatogenic cycle was achieved using periodic acid-Schiffs base (PAS) staining and the Leblond-Clermont system of classification. The study by Morales *et al.* (1989) was undertaken using animals with stage synchronised testis. This was achieved by a period of vitamin A depletion to allow regression of spermatogenesis followed by vitamin A repletion. The tubules are synchronised to a few related stages of the seminiferous epithelium and it is thought that the function of each stage is unchanged from the normal testis. However, the stage specificity of SGP-2 mRNA expression observed using these animals appears to be more pronounced than that seen previously with normal animals (Morales *et al.*, 1987). Despite these differences it can be concluded that mRNA for both SGP-1 and SGP-2 are heavily expressed by Sertoli cells at all stages of the spermatogenic cycle with only small changes in levels of expression occurring at different stages.

Further evidence for the constitutive expression of the sulphated glycoproteins, SGP-1 and SGP-2, was provided in these studies in which germ cell depletion was seen to have no effect on Sertoli cell expression of the messenger RNAs. The level of expression of SGP-2 mRNA has been shown to be unchanged with germ cell depletion caused by testosterone withdrawal from the adult rat *in vivo* (Roberts *et al.*, 1992). The addition of germ cells to immature Sertoli cells in culture has also been shown to have no effect on the levels of SGP-1 and SGP-2 mRNA (Stallard & Griswold, 1990). However, the addition of round spermatids to immature Sertoli cells in culture has been shown to increase the secretion of SGP-2 into the culture medium (Onoda & Djakiew, 1990). Taken together these results suggest that the presence of germ cells may have an effect on Sertoli cell production of the sulphated glycoproteins but that they do not affect the total amount of cellular mRNA. The lack of effect of germ cells at the transcriptional level makes these Sertoli cell products ideal controls for the study of germ cell influence on other Sertoli cell mRNAs.

The studies outlined in this chapter have provided further evidence for a cyclical function of Sertoli cells. Expression of mRNA for all the products studied, with the exception of SGP-2, was shown to vary with the stage of the spermatogenic cycle. This indicates that the proteins will probably also be produced in a cyclical manner and at such times as to meet the demands of the developing germ cells as they progress through the spermatogenic cycle. Numerous recent studies have shown that the cycle of Sertoli cell function is regulated, at least in part, by the complement of germ cells with which the Sertoli cell is associated at each stage of the spermatogenic cycle (for review see Sharpe, 1993). The influence of germ cells in modulating specific Sertoli cell mRNA expression for transferrin and CP-2 has also been shown in this chapter. Failure to identify germ cell regulation of other Sertoli cell products studied does not rule out paracrine regulation as it is possible that germ cells exert an effect other than at the level of transcription. In addition, in the seminiferous epithelium each Sertoli cell is associated with more than one germ cell type. Specific germ cells may exert different influences on the Sertoli cell and may modulate the production of Sertoli cell products according to their own needs. This may complicate interpretation of the results of germ cell depletion on Sertoli cell function as the absence of one germ cell type may be compensated for by signals from other germ cells. There are indications that this may be the case at least for some Sertoli cell products such as CP-2 (see chapter 6 for further discussion). These studies do however provide preliminary evidence for germ cell regulation of Sertoli cell mRNA levels and led to the further studies described in the following chapters.

5. Transferrin Production by the Sertoli Cell

5.1. Introduction

Iron is an essential nutrient required for the growth and survival of all mammalian cells. The main iron transporting protein in blood is transferrin, a 76kDa serum glycoprotein. It has been shown that proliferating cells *in vitro* have an absolute requirement for transferrin but this is thought to be indirectly due to their need for a supply of iron for DNA synthesis (Barnes & Sato, 1980; Laskey *et al.*, 1988). Transferrin is synthesised mainly by liver epithelial cells but Sertoli cells have also been shown to synthesise and secrete transferrin (Skinner & Griswold, 1980). The cDNA for liver transferrin has been cloned (Huggenvik *et al.*, 1987). Receptors for transferrin have been shown on spermatocytes and early spermatids in the seminiferous epithelium (Brown, 1985). The progression of germ cells through the spermatogenic cycle is dependent on a supply of iron and transferrin is thought to function to provide germ cells with this iron (Huggenvik *et al.*, 1984). The synthesis of transferrin by Sertoli cells is thought to be necessary as serum transferrin is unable to enter the seminiferous epithelium due to the presence of the blood-testis barrier.

Transferrin has been measured by a number of investigators as a marker of Sertoli cell function. The means by which transferrin production by Sertoli cells is regulated is of interest because of the importance of iron in the process of spermatogenesis. *In vitro* secretion of transferrin by Sertoli cells has been demonstrated to be influenced by a number of hormones and growth factors. Addition of FSH, retinol and insulin to cultured immature or mature Sertoli cells was shown to stimulate transferrin secretion (Skinner *et al.*, 1989a; Karzai & Wright, 1992). EGF and insulin have also been shown to act synergistically to stimulate transferrin secretion while interleukin 1α reduced transferrin secretion by mature Sertoli cells in culture (Karzai & Wright, 1992). Regulation of Sertoli cell transferrin synthesis by particular germ cells has also been shown in several studies. The addition of pachytene spermatocytes and round spermatids to immature Sertoli cells *in vitro* stimulated transferrin secretion (Le Magueresse *et al.*, 1988) and the level of transferrin mRNA expression has been shown to increase in Sertoli cells co-cultured with germ cells (Stallard & Griswold, 1990).

Initial studies presented in Chapter 4 of this thesis implicated round spermatids in the control of transferrin mRNA expression in the adult rat *in vivo*. The present chapter presents a more detailed study of the possible involvement of germ cells in the regulation of Sertoli cell transferrin mRNA expression. Interpretation of those and previous findings has been complicated by the presence of an additional mRNA transcript (hemiferrin) with sequence homology to the 3' end of transferrin, which appears to be expressed only within the germ cells (Stallard *et al.*, 1991).

5.2. Experimental Procedures

5.2.1 cDNA probes

All initial studies were carried out using a 688bp cDNA for the 3' region of rat transferrin subcloned into SP64 (Pharmacia) obtained from Dr. Steve Sylvester (University of Washington; Huggenvik *et al.*, 1987). Permanent stocks were prepared as described in Chapter 4. A further plasmid (pGem-4Z) containing a 705bp cDNA for the 5' region of rat transferrin was obtained from Dr. Kenneth Roberts (Johns Hopkins University, Baltimore, Maryland, USA; homologous to human transferrin cDNA, Uzan *et al.*, 1984). This was transformed into competent JM109 E.coli by heat shock and glycerol stocks taken again as described in Chapter 4.

5.2.2 Preparation of cDNA probes for Northern blot

3' Transferrin Probe. The cDNA insert was cut out of the plasmid by digestion with Hinc II and Pst I under normal reaction conditions (see chapter 3, section 8.1). The insert was separated from the cut vector by electrophoresis through a 1.2% Nusieve agarose gel (Seakem, GTG) in 1 x TAE buffer (appendix I) , the DNA was visualised by viewing the gel under UV light and the insert cut out of the gel with a scalpel.

DNA was eluted from the agarose using a silica based matrix provided in the MERmaid kit (Stratech Scientific Ltd.). Briefly, the gel piece containing the transferrin cDNA was melted at 50°C in an Eppendorf tube containing 3 volumes of a high salt solution and an appropriate amount of the silica matrix (Glassfog; approximately 8μl per μg DNA). Adsorption of DNA to the Glassfog was allowed to continue for a further 5min at room temperature before centrifugation (13,000rpm for 2min) to pellet the Glassfog. The supernatant was discarded, the pellet washed several times with ethanol and all traces of wash were removed. DNA was eluted by resuspending the matrix in water and incubating at room temperature for 5min, sample was centrifuged, the matrix was pelleted and the supernatant containing DNA was removed and saved.

5' Transferrin Probe. The cDNA was amplified from plasmid DNA by PCR using T7 and SP6 primers with an annealing temperature of 45°C and reaction conditions as described in chapter 3, section 6.1. Amplified cDNA was isolated from the PCR reaction mix using Chroma Spin +TE-100 columns (Clontech).

cDNAs were labelled with ^{32}P-dCTP in a random primed transcription reaction (see chapter 3, section 6.2). Northern hybridisation to samples from testis (chapter 3, sections 3 and 7.1) was carried out overnight at 65°C in 15% formamide buffer and post hybridisation washes were as previously detailed (see chapter 3, section 7).

5.2.3 *In situ* hybridisation

3' transferrin probe. Plasmid was linearised with Pst I and labelled with ^{35}S-UTP to give a riboprobe antisense to transferrin mRNA. Transcription was catalysed by SP6 RNA polymerase (see chapter 3, section 8).

5' transferrin probe. Plasmid was linearised with Eco RI or Hind III and transcription was catalysed by either T7 or SP6 polymerase to give antisense and sense riboprobes, respectively (see chapter 3, section 8).

All *in situ* hybridisation reactions were carried out as described for radioactive *in situ* hybridisation in chapter 3, section 9. RNase pretreatment was used as a control to determine non-specific hybridisation when using the 3' transferrin probe. Sections were treated with 20μg/ml RNase in RNase buffer at 37°C for 30min before addition of prehybridisation buffer to the sections.

5.2.4 RNA isolation from enriched Sertoli cell and germ cell fractions

Dissection medium. M199 media (Gibco) with 25mM Hepes, Earle's salts and L-glutamine to which was added 0.2% D-(+)-glucose (Sigma), 0.1% BSA (Fraction V; Sigma), 2,000U penicillin-streptomycin (10,000IU/ml; Gibco) and 4,000U DNase (20,000U/ml; Sigma), all at pH 7.4.

Elutriation medium. Dissection medium with 12,500U heparin (Sigma) and 1mM EDTA.

Cells were isolated from the testis of normal adult male rats essentially as described elsewhere (Meistrich *et al.*, 1981) but with some modifications. Testis were decapsulated, chopped up in a small volume of dissection medium and both testis from each rat were placed in 50ml falcon tubes containing 10ml dissection medium with 150μl trypsin (25mg/ml of 7,500-10,000U/mg; Sigma). This was incubated at 37°C in a shaking water bath for 30min, 15ml dissection medium added, the tubes inverted 40 times with a rotational movement and the tubules allowed to settle before the medium was removed and discarded. A further 5ml of dissection medium containing 2mg/ml collagenase (Batch 4196; Worthington Biochemical Corporation, Cambridge Biosystems, Cambridge, UK.) and 0.44% trypsin inhibitor (Sigma) was added to the tubules and the incubation was continued at 37°C for 45min in a shaking water bath. Dissection medium (10ml) was again added, the tubes rotated approximately 20 times and undigested tubules were allowed to settle before the medium was removed, filtered through a 60μm gauze and saved. Collagenase treatment was repeated if necessary on undigested tubules.

Table 1. Isolation of enriched cell fractions.

Fraction	Rotor speed -rpm	Pump flow rate-ml/min	Cell type
Load	3500	10	Type B spermatogonia, preleptotene spermatocytes, late spermatids
1	3000	20	Type A and I spermatogonia, leptotene spermatocytes, RBs and late spermatids
2	2000	30	Early and late spermatids, early pachytene and zygotene spermatocytes
3	1000	40	Pachytene spermatocytes and Sertoli cell clumps
4	0	70	Sertoli cells and late spermatids

Rotor speed and pump flow rate used to isolate and separate cells of the seminiferous epithelium.

Cells were pelleted at 800g for 10min at room temperature, resuspended in a minimal volume (approx. 5ml) of the supernatant and taken up in a 10ml syringe. The elutriator (Beckman model J-6B) was prepared by washing with distilled water followed by elutriation media. Cells were injected into the elutriation system with the pump off and an elutriator speed of 3500rpm, the pump was switched on to a speed of 10ml/min and 100ml of medium containing cells collected. This was the load fraction. Subsequent fractions containing different cell types were collected by changing the pump flow rate and the elutriator speed (Table 1).

Collected fractions were centrifuged at 800g for 10min at room temperature to pellet the cells. For RNA extraction pelleted cells were resuspended in 2ml of solution D and passed through a 16 gauge needle to rupture the cells. Preparation of total RNA was continued as described in chapter 3, section 3.

5.3. Results

5.3.1 Localisation and stage specific expression of transferrin mRNA

In situ hybridisation performed using the 3' transferrin probe showed a positive signal localised specifically to cells within the seminiferous epithelium (Fig.1a). The diffuse signal meant precise identification of the cell type/s in which mRNA/s recognised by this probe was expressed was not possible.

The level of signal detected varied between different seminiferous tubules depending on the germ cell complement (Fig.1b). Quantification of the amount of expression in tubules at each stage of the spermatogenic cycle was carried out by counting the number of silver grains in at least five tubules at each stage in sections from each of two rats using the Cue 2 image analysis software package (Olympus). Highest levels of expression were detected at stages XIV and I of the spermatogenic cycle with lowest levels at stage VIII (Fig.2).

5.3.2 Regulation of transferrin mRNA expression by germ cells

In situ hybridisation with the 3' riboprobe on testis sections from rats at selected time points after MAA treatment was carried out to determine the effect of germ cell depletion on transferrin mRNA expression. At 3d after MAA treatment, pachytene spermatocytes were depleted from all stages of the spermatogenic cycle with the exception of stages I and VIII-XI. Stages VIII-XI had a full complement of germ cells while round spermatids were depleted from stage I tubules. Transferrin mRNA expression did not appear to be significantly changed at any stage of the spermatogenic cycle with the exception of stage I which showed a lower level of expression compared to controls (Figs.3a & 3b). At 7 days after MAA treatment tubules at stages II-VI lacked round spermatids while stages VII-XIII were depleted of pachytene or diplotene spermatocytes. A decrease in transferrin mRNA expression was obvious in stage II-VI tubules while there was no change at any other stage when compared to controls (data not shown). A decrease in expression was also seen in tubules at stages I-VII at MAA+14d when round spermatids were depleted from these tubules (Fig.3c). The depletion of step 9 to step 14 spermatids from other stages of the spermatogenic cycle at this time point did not appear to influence transferrin mRNA levels. At 21d after treatment elongating spermatids were depleted from tubules at stages VIII-XIII and I-VI of the spermatogenic cycle. The absence of these germ cell types did not appear to influence expression of the mRNA (Fig.3d). However, round spermatids were

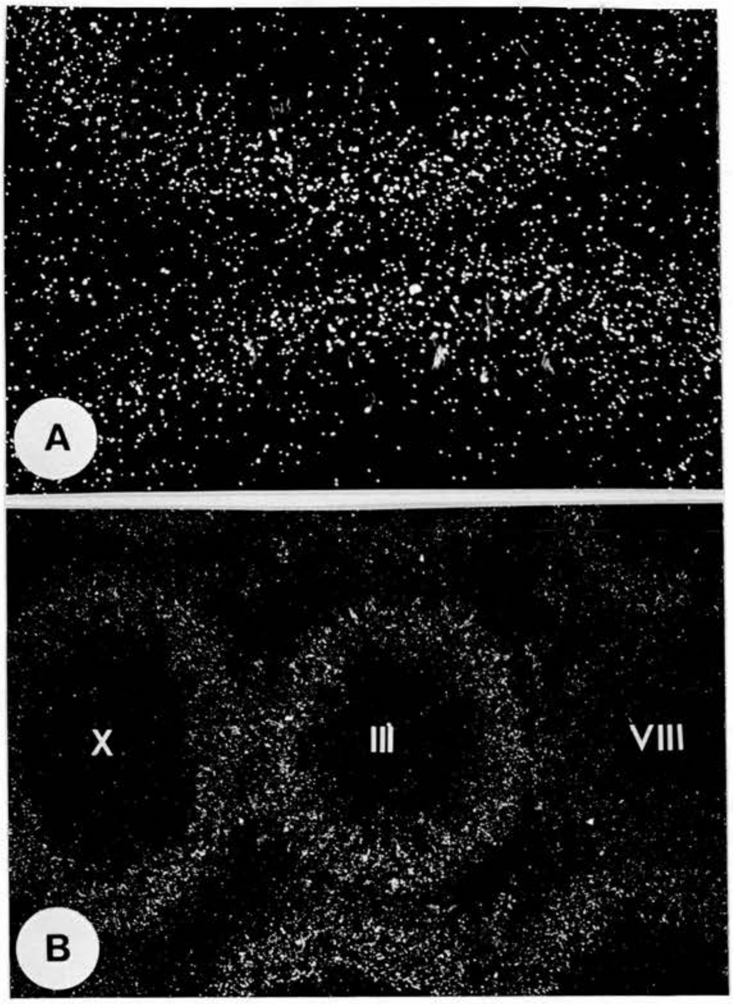


Figure 1. Localisation and stage specific expression of transferrin mRNA. Darkfield photomicrographs of the localisation (A) and stage specific expression (B) of transferrin mRNA in control rat testis using an antisense ^{35}S-UTP labelled riboprobe. x227 and x90 magnifications, respectively.

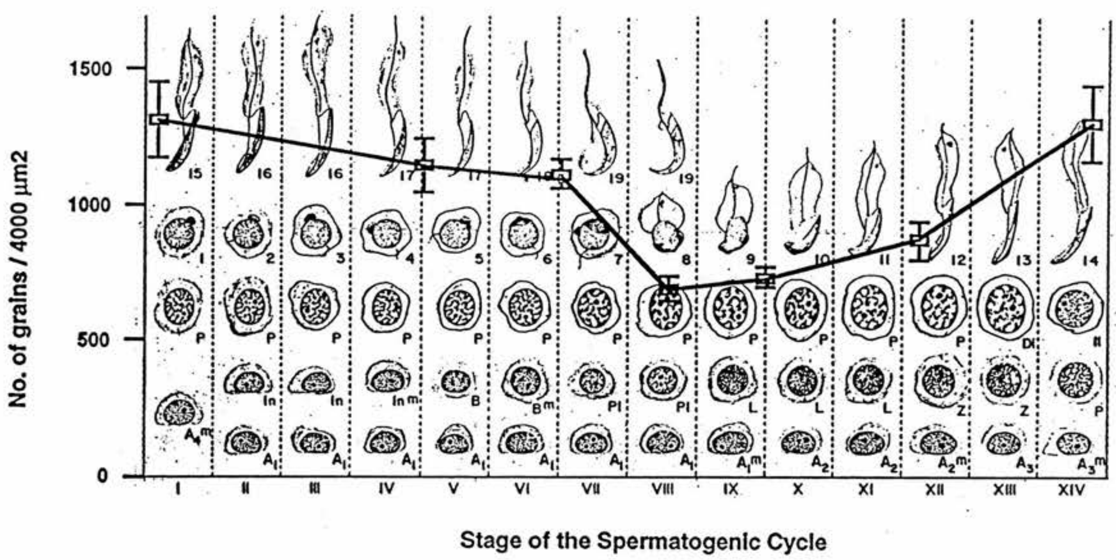
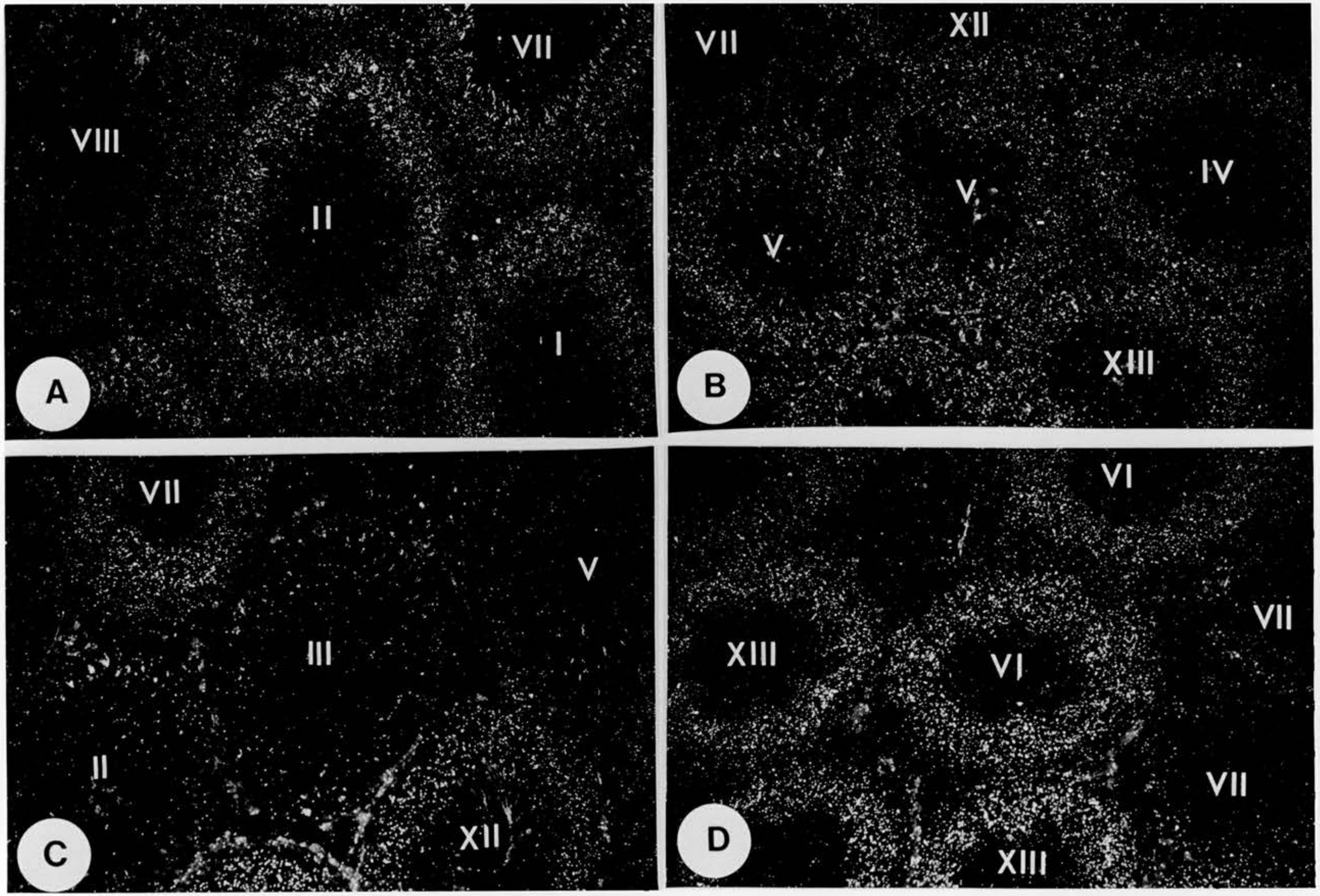


Figure 2. Stage specific expression of transferrin mRNA. Levels of transferrin mRNA at each stage of the spermatogenic cycle as shown by radioactive *in situ* hybridisation. Levels were calculated by counting the number of silver grains in at least five tubules at each stage in testis sections from each of two normal rats. Profile of mRNA expression is overlaid on a diagram of the spermatogenic cycle to show the germ cell complement at each stage in association with transferrin mRNA levels.

Figure 3. Effect of germ cell depletion on transferrin mRNA levels. Darkfield photomicrographs of transferrin mRNA expression in (A) control rat testis, (B) MAA + 3d rat testis, (C) MAA + 14d rat testis, and (D) MAA + 21d rat testis using a ^{35}S-UTP labelled riboprobe. x90 magnification.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depleted from stage VII tubules and expression at this stage was reduced compared to controls (not shown). Finally, there was no significant change in the level of expression of transferrin mRNA at 28d after MAA when elongating spermatids were depleted from stage I-IV tubules only, or at MAA + 42d when all tubules had a full complement of germ cells.

Quantification of the changes observed using the image analysis software was carried out for two stages of the cycle; stage VIII at which the lowest levels of expression were detected in control rats and stage I where maximal expression was observed. Expression of transferrin mRNA in stage VIII tubules was not significantly changed at any of the time points after MAA treatment, even at MAA + 7d when pachytene spermatocytes were depleted from the tubules (Fig.4a). However, the signal detected in stage I tubules was observed to decrease significantly at both 3d and 14d after MAA treatment, times at which round spermatids were depleted from tubules at this stage (Fig.4b). The depletion of elongate spermatids from stage I tubules 21d after MAA treatment did not significantly change transferrin mRNA levels.

5.3.3 Identification of transferrin transcripts in testicular tissue

Northern blot analysis with the 3' transferrin probe detected two transcripts in total RNA isolated from control rat testis, one of 2.6kb and the other more abundant transcript of approximately 0.9kb (Fig.5, lane 1). The 2.6kb transcript was detected only in total testis and in Sertoli cell RNA while the 0.9kb transcript was found in RNA isolated from fractions enriched in germ cells but was absent in the Sertoli cell fraction (not shown). A probe specific for the 5' region of transferrin detected only one transcript in the testis. This was approximately 2.6kb and was seen in total testis RNA (Fig.5, lane 2) and in RNA isolated from an enriched Sertoli cell fraction (not shown). This transcript was not detected in any germ cell fraction (not shown).

A sequence alignment of the 3' and 5' probes with transferrin cDNA sequences present in the database was carried out. The 3' probe was found to have significant homology to human transferrin and to hemiferrin (Stallard *et al.*, 1991), a transcript detected in round spermatids (Fig.6). The 5' probe had homology only to human transferrin cDNA (Uzan *et al.*, 1984) and did not cross react with hemiferrin.

Membranes containing RNA isolated from rat testis at all times after MAA treatment were hybridised to the transferrin cDNA probe specific to the 5' end of the complete mRNA. Using this method no significant change in the level of expression of the 2.6kb transcript was observed at any time point after treatment (data not shown). Using the 3' transferrin probe the level of expression of the 0.9kb transcript was observed to decrease significantly at 7d and 14d when round spermatids were the main

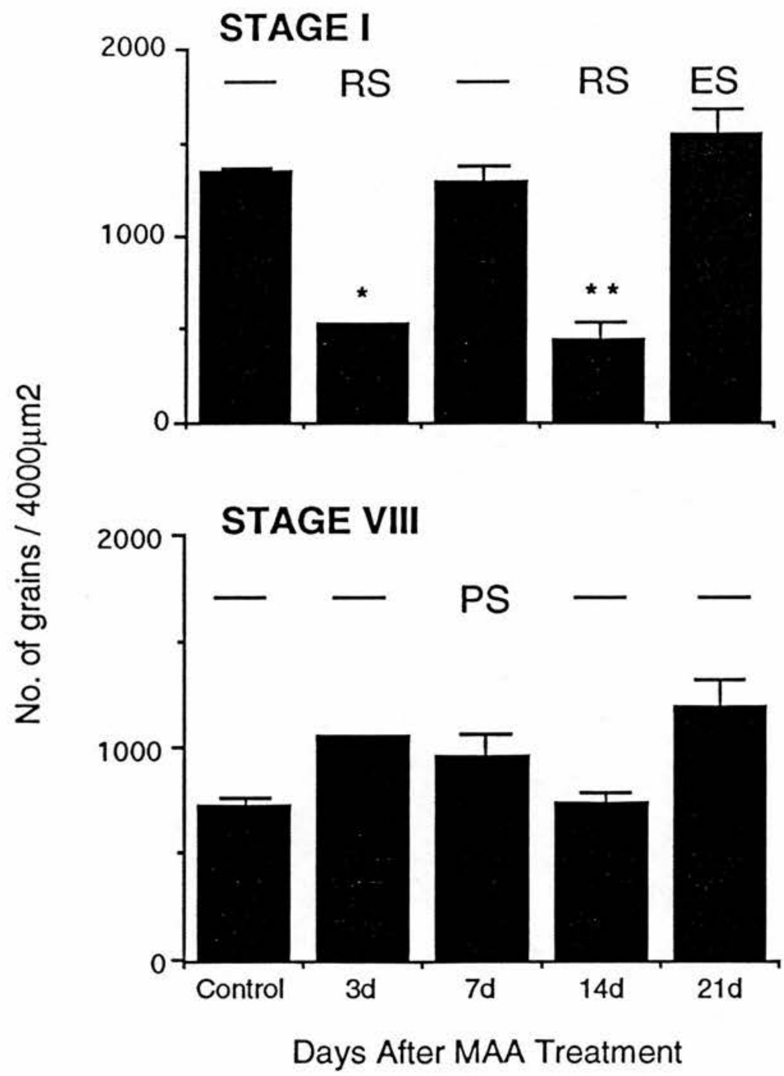


Figure 4. Quantification of change in transferrin mRNA levels due to germ cell depletion. Levels of transferrin mRNA measured in stage I and stage VIII tubules calculated by counting silver grains using an image analysis system. Cell types missing from each stage are shown above the graphs. PS=Pachytene spermatocytes, RS=Round spermatids, ES=Elongate spermatids, - =no germ cells missing. *$p<0.05$, **$p<0.01$.

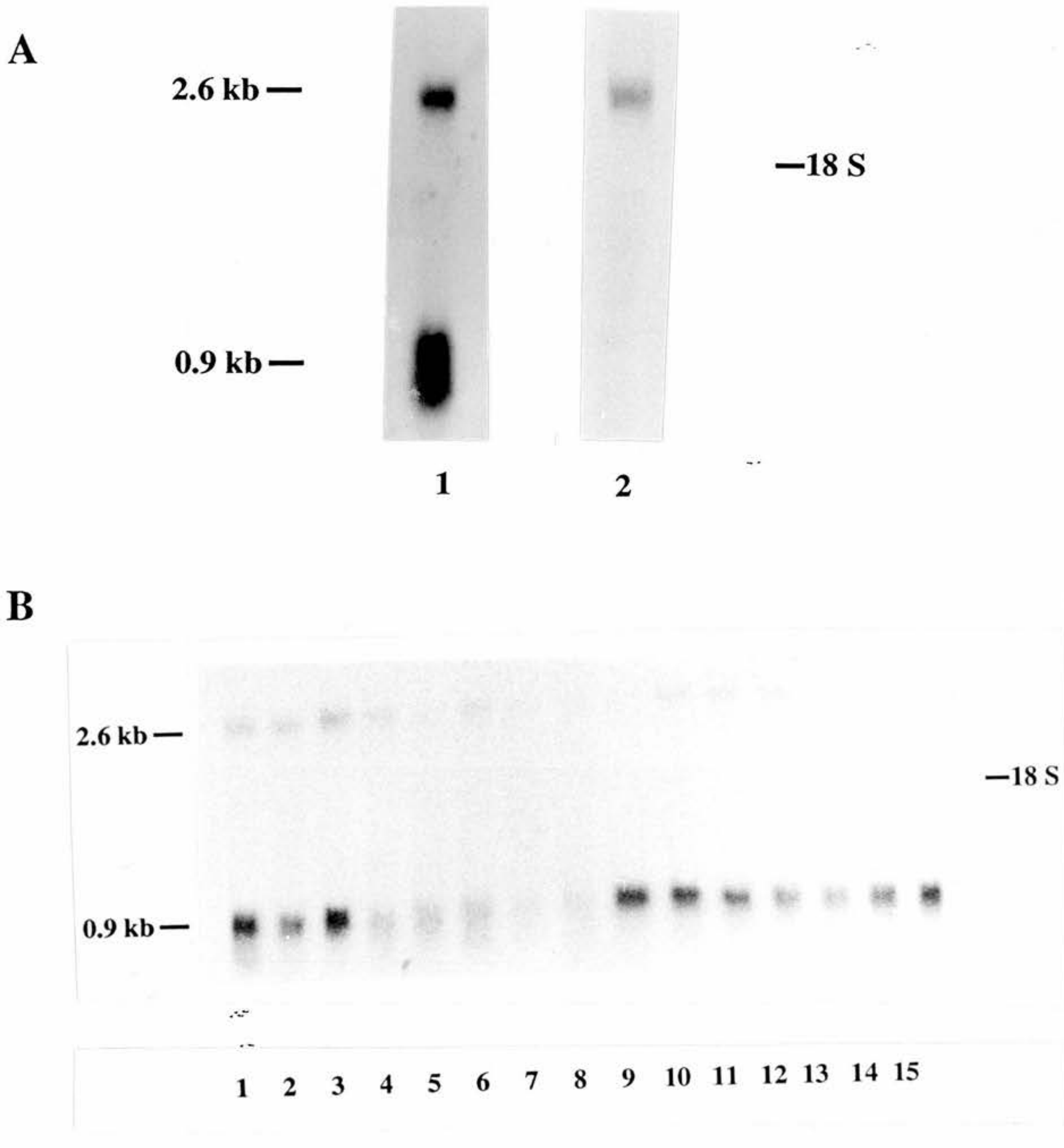


Figure 5. Northern blot analysis of transferrin mRNA expression. RNA samples in panel A are from control rat testis and in panel B from control rat testis (lanes 1, 2 &15), or testis from animals 3d (3&4), 7d (5&6), 14d (7&8), 21d (l9&10), 28d (11&12) and 42d (13&14) after MAA treatment. Lanes were loaded with 15µg total RNA. The membranes were hybridised with ^{32}P-labelled 3' transferrin cDNA (panel A, lane 1 and panel B) or 5' transferrin cDNA (panel A, lane 2) and exposed to X-Omat AR film for 48 hours.

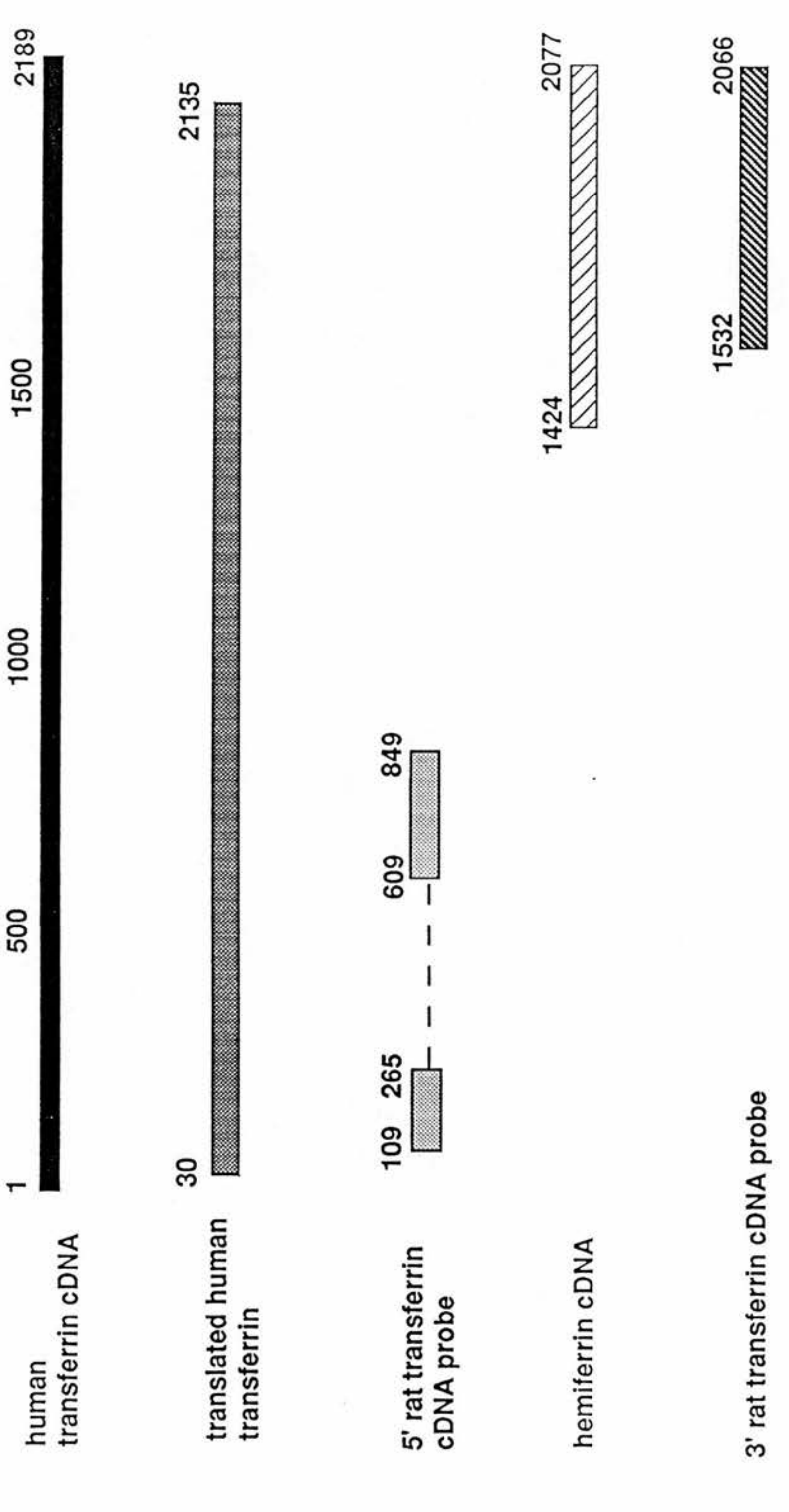


Figure 6. Sequence alignment of transferrin probes. Diagram outlining the areas of homology between the 3' and 5' transferrin probes used in the present study and the hemiferrin and human transferrin full length cDNAs. Portion of human transferrin cDNA translated is also illustrated. Dotted line in 5' transferrin cDNA indicate areas of cDNA which have not been sequenced but are assumed to align.

germ cell type depleted from the tubules (Fig. 5B). *In situ* hybridisation with the 5' probe which was specific for Sertoli cell transferrin mRNA was unsuccessful.

5.4. Discussion

The aim of the studies described above was to investigate the possible involvement of germ cells in the modulation of Sertoli cell transferrin mRNA expression. Previous studies *in vitro* have demonstrated an influence of germ cells either on transferrin mRNA levels or on secretion of the protein by Sertoli cells. Addition of a crude germ cell fraction to immature Sertoli cells in culture was shown to stimulate transferrin mRNA levels (Stallard & Griswold, 1990). This activity was shown to be associated mainly with conditioned medium from pachytene spermatocytes and partial characterisation of the components responsible for this stimulatory activity revealed several proteins with molecular weights of approximately 10-30kDa. Inhibition of protein synthesis and RNA transcription demonstrated that both transcription and translation were necessary for the germ cell stimulation of transferrin mRNA expression. Stimulation of transferrin secretion by immature Sertoli cells in culture has also been demonstrated on addition of pachytene spermatocytes or round spermatids (Le Magueresse *et al.*, 1988). This stimulation was shown to be due mainly to secreted factor/s from these cells as activity was maintained on addition of germ cell conditioned media but not when germ cell membranes were added to the Sertoli cell cultures. Using a bicameral chamber system, stimulation of Sertoli cell transferrin secretion by addition of round spermatid conditioned media was seen to be predominantly in the apical direction (Onoda & Djakiew, 1990). Secretion in this direction would be towards the germ cells *in vivo* and would be consistent with round spermatids directing Sertoli cell transferrin secretion for their own use.

It is generally accepted that influences on Sertoli cell function demonstrated using in *vitro* systems with immature Sertoli cells are not necessarily indicative of what will occur in the adult animal *in vivo*. Therefore it is necessary to confirm results obtained *in vitro* using more physiological experimental conditions. In accordance with this, involvement of germ cells in the control of transferrin mRNA expression has also been shown by *in vivo* studies. Hypophysectomy of adult rats resulted in regression of spermatogenesis and a corresponding decline in the level of transferrin mRNA in Sertoli cells (Roberts *et al.*, 1991). This was reversed by repopulation of the seminiferous epithelium with germ cells following testosterone treatment. A similar study demonstrated that depletion of germ cells from the seminiferous epithelium of adult rats due to testosterone withdrawal *in vivo* was accompanied by a decrease in transferrin mRNA expression (Roberts *et al.*, 1992). This decline in transferrin mRNA did not occur initially on depression of testosterone levels but was only observed when spermatogenesis began to fail. However, these studies did not identify individual germ cell types important for the regulation of transferrin gene expression.

Preliminary results reported in this chapter in which germ cell depletion occurred *in vivo* following MAA treatment has shown possible modulation of Sertoli cell transferrin production specifically by round spermatids, with the absence of either pachytene spermatocytes or elongate spermatids appearing to have no effect on this function of Sertoli cells. However, the cDNA probe directed against the 3' region used to detect the message in Sertoli cells by *in situ* hybridisation was shown by Northern blotting to cross-react with a transcript present in RNA from germ cells. Studies published by Stallard and co-workers (1991) have identified this transcript as hemiferrin and shown that its cDNA has homology to the 3' end of transferrin. It has to be assumed that the probe will also cross react with this mRNA species during the *in situ* procedure resulting in hybridisation both to Sertoli cell transferrin mRNA and to hemiferrin mRNA in round spermatids. In the present study, *in situ* hybridisation with this 3' transferrin probe showed silver grains localised over the seminiferous epithelium from the basal surface to the tubule lumen. This pattern of grains is not typical of what we would expect from a mRNA localised only in Sertoli cells (Millar *et al.*, 1993). Sertoli cell products such as CP-2, ABP, SGP-1 and SGP-2 all showed a similar pattern when *in situ* hybridisation was used to detect their transcripts; grains were localised mainly around the base of the seminiferous epithelium (chapter 4 of this thesis; Maguire *et al.*, 1993). It was therefore assumed that hemiferrin mRNA is being detected by *in situ* hybridisation with this probe. Using Northern blot analysis with a probe specific for the 5' end of transferrin which did not cross react with hemiferrin, ransferrin mRNA in total testis RNA appeared to remain constant at all time points after MAA treatment. The level of hemiferrin mRNA decreased at 7 and 14 days after MAA treatment demonstrating that the treatment had been successful and germ cells were depleted from the tubules. *In situ* results using the 3' probe in which positive signal was reduced following MAA are therefore assumed to reflect loss of germ cell transcripts rather than regulation of Sertoli cell specific mRNA.

Stage specific expression of transferrin mRNA by Sertoli cells in the seminiferous epithelium has been reported (Morales *et al.*, 1987; 1989). Expression was shown to be highest at stages XIII-XIV of the cycle and lowest at stages IX-X by *in situ* hybridisation. This pattern of expression closely resembles that reported in the present chapter with maximal expression at stages XIV-I and lowest levels at stages

VIII-IX of the spermatogenic cycle. These studies will probably be detecting two transcripts in the testis, hemiferrin mRNA and transferrin mRNA, as the same 3' cDNA was used in all studies. However when *in situ* hybridisation was performed with a biotinylated cRNA probe transcribed from a 3' transferrin cDNA identical to the one used in the present study staining was observed only within the Sertoli cell cytoplasm (Morales *et al.*, 1987). Our studies have suggested that the use of riboprobes for *in situ* hybridisation containing a digoxigenin or biotin label is much less sensitive than using ^{35}S-UTP labelled riboprobes (Millar *et al.*, 1993). Hybridisation conditions need to be adjusted to take account of steric interference caused by the digoxigenin or biotin molecules incorporated along the length of the probe. This may mean that the biotinylated transferrin riboprobe used by Morales and co-workers (1987) detected only the Sertoli cell transcript and not that for hemiferrin because the homology is not complete and the probe was washed off under stringent conditions of hybridisation. However quantification data to determine the stage specific expression of transferrin mRNA in the study by Morales and co-workers (1987) was based on data using a tritiated cRNA probe. Although the authors presumed this to be due to localisation to the Sertoli cells alone data from the current study would suggest their results would be complicated by hybridisation to hemiferrin within germ cells. A study using Northern blot analysis of RNA isolated from microdissected staged tubules probed with a transferrin specific cDNA has been carried out (Roberts & Griswold, 1990). The results showed very slight stage specific differences in the level of expression, with maximal levels at stages XIII-I, indicating that the changes observed in other studies may be due to Sertoli cell transferrin mRNA expression. The present study attempted to clarify these results by the use of a probe directed against the 5' region of rat transferrin cDNA and which was entirely specific for the Sertoli cell transcript. Unfortunately, *in situ* hybridisation with this probe on testicular tissue has been unsuccessful to date but this experiment would finally establish the true stage specificity of transferrin mRNA expression and would demonstrate potential germ cell regulation.

Transferrin has been detected in the rat testis by binding of iodinated transferrin and by immunostaining. The use of isolated cells indicated that ^{125}I-transferrin bound mainly to fractions enriched in pachytene spermatocytes or to Sertoli cells isolated from immature rat testis (Holmes *et al.*, 1983). However, immunostaining to localise transferrin in the adult rat testis showed the protein mainly associated with spermatids from stage 1 to stage 17 of development (Sylvester & Griswold, 1984). These conflicting results could be due to the different techniques used. The transferrin receptor has been localised by indirect immunofluorescence to dividing spermatocytes and early spermatids (Sylvester & Griswold, 1984). This pattern of localisation of the receptor has also been shown by direct immunostaining using an antibody specific for rat transferrin receptor (Brown, 1985). This study also showed the receptor to be present on Sertoli cells of immature rats and in the adult rat testis which was enriched in Sertoli cells due to irradiation of the animal *in utero*. These studies together indicate that transferrin may be utilised by spermatocytes and early spermatids but does not seem to be necessary for the development of mature spermatids. It is known that iron is essential for DNA synthesis and cell proliferation and this would account for the transferrin requirement of mitotically and meiotically dividing germ cells (Laskey *et al.*, 1988). The presence of transferrin in round spermatids could be due to their need for iron in their electron transport proteins as these cells are thought to be actively involved in mitochondrial biosynthesis (Hecht & Kennington, 1983). The possible regulation of Sertoli cell transferrin production by spermatocytes and early spermatids demonstrated in several studies may indicate that the germ cells are directing their own development by regulation of the amount of iron delivered to them.

In conclusion, the present study has shown that results of previous studies concerning the stage specific expression of transferrin mRNA may have been interpreted incorrectly. The presence of a mRNA with strong homology to transferrin which is expressed in round spermatids must lead to caution in the interpretation of results where the transcripts cannot be distinguished. The failure of this study to demonstrate germ cell regulation of transferrin mRNA expression in Sertoli cells does not rule out the possibility of this being an important means of control. Evaluation of germ cell regulation was only possible with Northern blot analysis which is a relatively insensitive technique and *in situ* hybridisation is necessary to rule out any effect of germ cell depletion on transcription of the transferrin gene. Finally, it is possible that germ cell regulation of Sertoli cell transferrin production occurs after gene transcription but this has not been addressed in the present study.

6. Modulation of stage dependent expression of cyclic protein 2 by spermatids

6.1. Introduction

Cyclic protein 2 (CP-2) was first described by Wright and co-workers in 1983. It was shown in this study by 2D gel analysis that the pattern of protein secretion from seminiferous tubules in culture changed according to the stage of the spermatogenic cycle. The protein designated CP-2 was seen to be secreted maximally by tubules at stage VI, the level of secretion at this stage being at least 20 times greater than that seen at stage XII of the cycle. The cellular origin of this protein was established by analysis of media from isolated testicular cell cultures and it was found to be secreted by Sertoli cells. The protein was finally isolated from seminiferous tubule fluid and Sertoli cell culture media and was demonstrated to be a small hydrophobic glycoprotein (Wright & Luzarraga, 1986). The change in secretion of CP-2 with the cycle of the seminiferous epithelium was shown to be a consequence of a change in the rate of synthesis of the protein by Sertoli cells (Wright *et al.*, 1989).

CP-2 production by adult Sertoli cells in culture can be stimulated by a combination of FSH, retinol and testosterone but these investigators did not study the role of any other factors (Karzai & Wright, 1992). However, the dramatic stage-specific changes observed in the secretion of CP-2 made it a good candidate for potential germ cell regulation and worthy of more detailed study. A cDNA for CP-2 was recently isolated and comparison of this cDNA with known DNA sequences showed significant homology to the proenzyme form of cathepsin L, a cysteine protease (Erickson-Lawrence *et al.*, 1991). During the current investigations the published sequence was used to isolate a partial cDNA for CP-2 from a pool of total testis cDNA using PCR and a study of the germ cell regulation of CP-2 mRNA expression in the Sertoli cell was undertaken. Preliminary results have been reported in chapter 4 but the present chapter reports the results obtained from a more detailed study of the expression of CP-2 mRNA. An antibody directed against CP-2 was also obtained and was used for further studies on the modulation of CP-2 production at the level of the protein. The development of stage specific synthesis of CP-2 in immature animals was also investigated.

6.2. Experimental Procedures

The partial cDNA for CP-2 used as a template for probe preparation in the present studies was amplified and subcloned into Bluescript as described in chapter 4.

Northern blot and *in situ* hybridisation analysis were carried out as described in chapter 3.

6.2.1 Preparation of CP-2 antibody

An antibody specific for cyclic protein 2 was obtained from Dr. W. Wright, Johns Hopkins University, Baltimore (Wright, 1988). The antibody was raised in rabbit to purified rat CP-2 and was received as 2ml of lyophilised antiserum. This powder was resuspended in 4ml pure water and the IgG fraction of the antiserum isolated by fractionation on a protein A Sepharose column as follows. Serum was dialysed against 0.02M phosphate buffer, pH7.2 overnight at 4°C to remove salts. A disposable plastic column (BioRad) was packed with 1ml Protein A Sepharose (CL-4B; Sigma) and equilibrated with dialysis buffer. The column was attached to a fraction collector and pump (BioRad), the serum loaded and the absorbance of the flow through at 280nm was monitored. The column was washed with approximately 10ml phosphate buffer until the A_{280} was zero and the IgG eluted from the column with 0.58% acetic acid in 0.15M NaCl. Fractions of approximately 1ml were collected and those containing the IgG were pooled (ie. those fractions showing a peak of absorbance at 280nm).

CP-2 IgG fraction was adsorbed against bacterial lysates to prevent cross reaction with the basement membrane during immunostaining. Bacterial lysates were prepared as follows; Y1090 E.coli were cultured overnight in LB-broth (appendix I) containing 0.2% maltose and 10mM $MgSO_4$. Cells were centrifuged at 1600g and resuspended in 10mM $MgSO_4$. Tubes containing 500μl Y1090 to which were added 5 x 10^5 pfu lambda gt11 were incubated at 37°C for 15min, 10ml NZY top agar (0.9%; appendix I) was added and the cells plated onto 150mm NZY agar plates (1.5%; appendix I). These were incubated at 37°C for approximately 2h until pinprick plaques appeared, the plates overlaid with Hybond C nitrocellulose filters (Amersham) and the incubation continued overnight. Membranes were then removed and placed in a glass beaker.

The IgG fractions prepared above were diluted 1:50 with 1% BSA in TBS (0.01M Tris buffered saline, pH7.5) and adsorbed against the membranes at 4°C overnight. This procedure was repeated twice, the adsorbed antiserum was filtered with a 0.2μm millipore filter and stored in 0.1% sodium azide at -20°C. The titre of the CP-2 antibody was decreased by the adsorption procedure and so was concentrated by dialysis against solid polyethylene glycol (MW 6000) for 8h at room temperature before use.

6.2.2 Immunostaining

Immunostaining was carried out as described in chapter 3 using either CP-2 or SGP-1 (antibody kindly supplied by Dr. Steve Sylvester, Washington State

University; Huggenvik *et al.*, 1987) as primary antibody. CP-2 antibody was used undiluted after adsorption and PEG concentration while SGP-1 was used at a dilution of 1:1000 (diluted in 1:5 normal swine serum:TBS).

6.3. Results

6.3.1 CP-2 cellular localisation and stage specificity

The mRNA for CP-2 was localised by *in situ* hybridisation to the base of seminiferous tubules using a Digoxigenin-labelled riboprobe. When viewed under high power magnification the message was seen to be expressed in the cytoplasm of Sertoli cells (Fig.1a). The level of expression of CP-2 mRNA in the Sertoli cells varied considerably according to the stage of the spermatogenic cycle; highest levels were evident in Sertoli cells at stages IV-VII, whilst there was no expression detectable at other stages (Fig.1b).

An antibody specific for CP-2 was used to localise the protein to the Sertoli cells of the seminiferous epithelium (Fig.2a). The protein was seen to be present in Sertoli cell cytoplasm which was in contact with the heads of elongate spermatids. CP-2 was observed to be present at stages V-VIII of the spermatogenic cycle (Fig.2b) reflecting the stage specific expression of the mRNA for CP-2.

6.3.2 Development of stage specificity

Expression of CP-2 mRNA in immature rat testis was studied. *In situ* hybridisation showed expression of the transcript in 16 day old animals (Fig.3a). The signal was thought to be localised in Sertoli cells although it was not found along the base of the seminiferous tubules as in the adult rat testis. There did not appear to be a signal in all tubules indicating a degree of stage specific expression. CP-2 mRNA was also detected in the testis of day 23, day 32 and day 42 rats (Fig.3c-e). Definite stage specific expression was visible by day 23 and localisation of the signal began to look more like that seen in the adult rat testis, ie. basally located in the seminiferous tubule.

6.3.3 Northern blot analysis of CP-2 mRNA expression

Two CP-2 mRNA transcripts were detected in the testis as reported in chapter 4 and previously described by Erickson-Lawrence *et al.* (1991). The 1.7kb transcript was more abundant than the 2.2kb mRNA in the testicular samples while the 2.2kb transcript was more heavily expressed in total RNA prepared from kidney (positive control; Fig.4).

MAA treatment of animals led to the selective depletion of pachytene spermatocytes at all stages of the spermatogenic cycle except at early to mid stage VII.

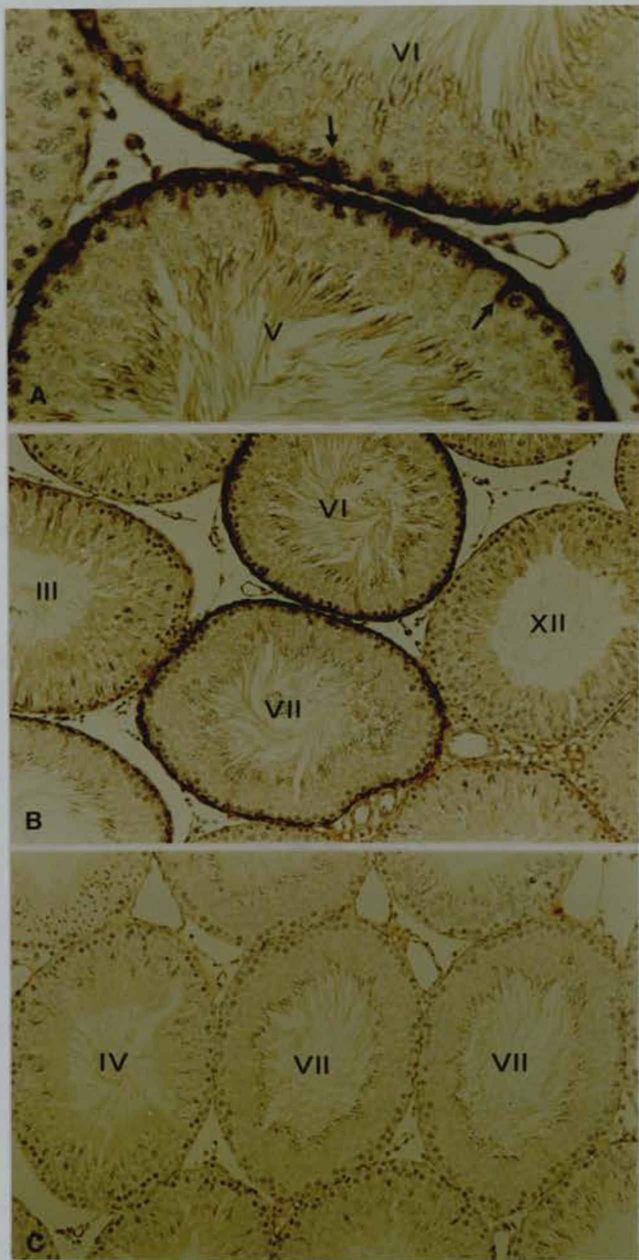


Figure 1. Localisation and stage specific expression of CP-2 mRNA. Brightfield photomicrographs of cross sections of perfusion fixed rat testis. The tissue was probed with DIG-labelled riboprobes antisense (A & B) or sense (C) to CP-2 mRNA. (A) Stages V and VI tubules from control testis expressing CP-2 mRNA and showing subcellular localisation of mRNA in the Sertoli cell (arrows). Magnification x 129.(B & C) Control rat testis showing stage specificity of CP-2 mRNA expression only when antisense riboprobe is used. Tubules expressing CP-2 mRNA are at stages IV-VII of the spermatogenic cycle. Magnification x52.

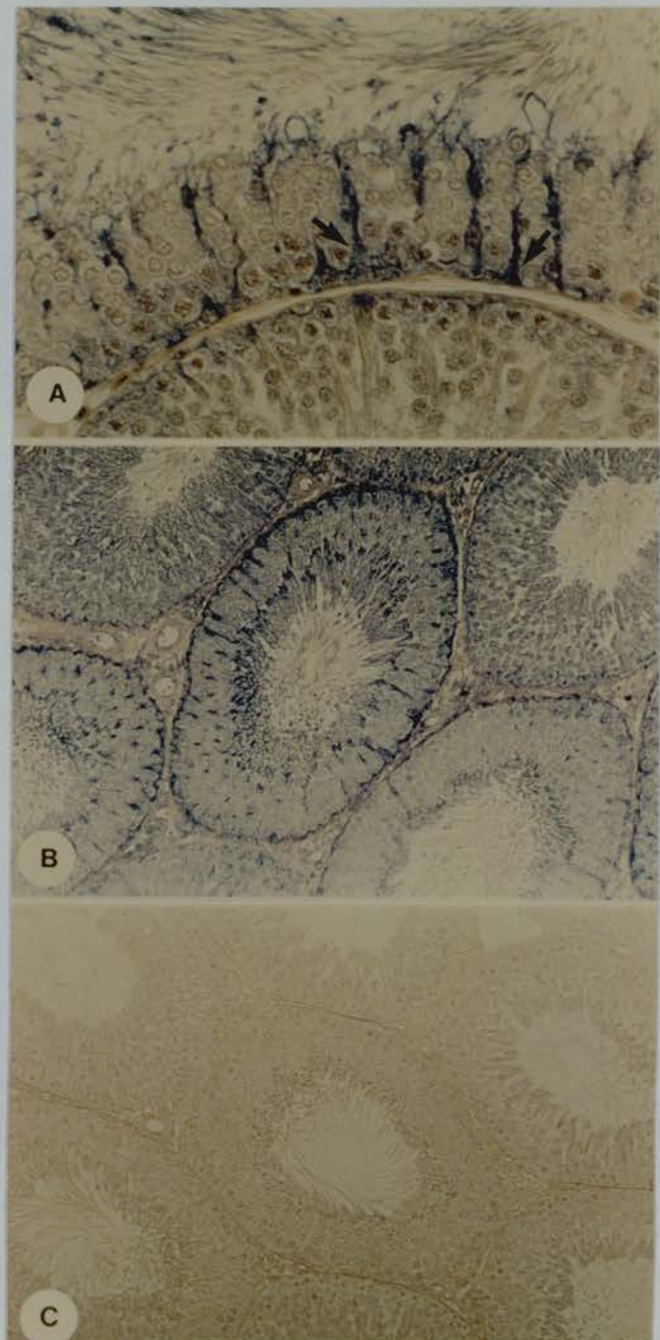
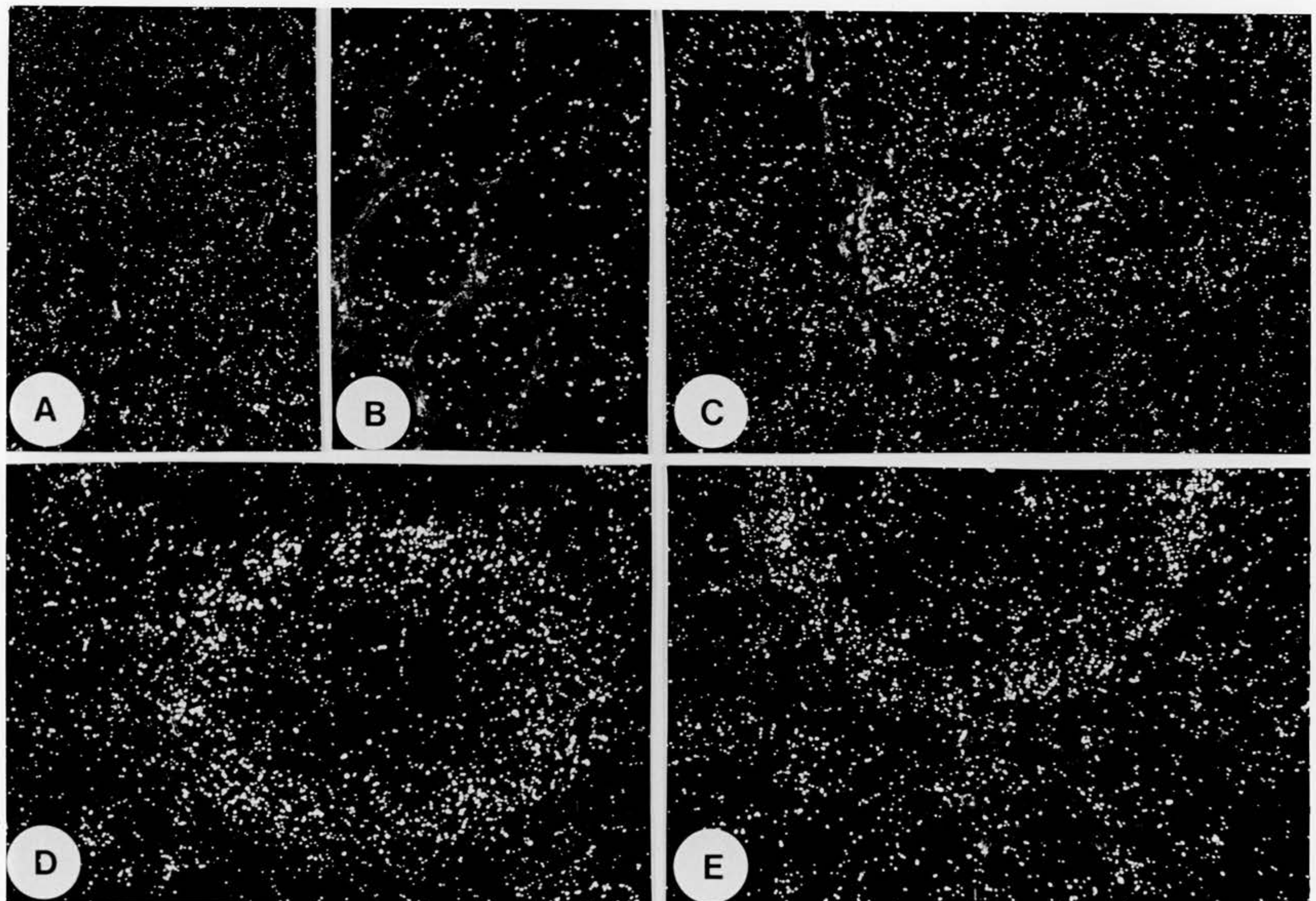


Figure 2. Localisation and stage specific expression of CP-2 protein. Brightfield photomicrographs of cross sections of perfusion fixed rat testis. The tissue was incubated with anti-rat CP-2 (A & B) or normal rabbit serum (C) as primary antibody. (A) Stage VIII tubule from control testis showing subcellular localisation of the protein in the Sertoli cell (arrows). Magnification x245. (B & C) Control rat testis showing stage specificity of CP-2 protein only when anti-CP-2 is used. Tubules in which the protein is detected are at stages IV-VII of the spermatogenic cycle. Magnification x227.

Figure 3. Developmental expression of CP-2 mRNA. Darkfield photomicrographs of cross sections of perfusion fixed rat testis. The tissue was probed with ^{35}S-labelled riboprobes antisense (A, C, D & E) or sense (B) to CP-2 mRNA. Sections are from animals 16d (A & B), 23d (C), 32d (D) and 42d (E) old and show expression of CP-2 mRNA as early as 16d after birth with an increase in the level of expression as the animal matures.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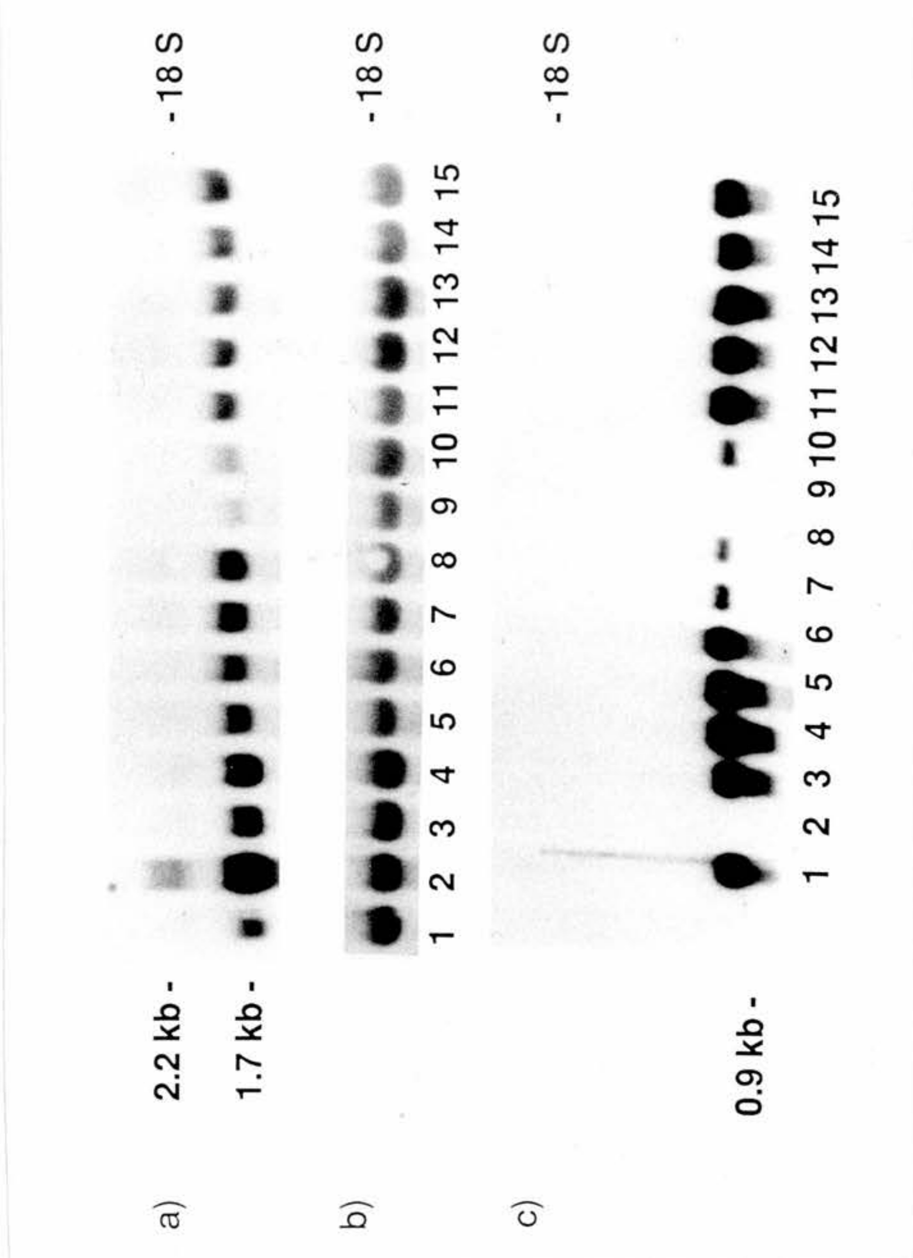


Figure 4. Northern blot analysis of CP-2 mRNA expression. Samples are from a control rat testis (lanes 1 and 15), kidney (lane 2) and testes from each of two animals administered a single oral dose of methoxyacetic acid 3, 7, 14, 21, 28 or 42 days previously (lanes 3-4, 5-6, 7-8, 9-10, 11-12 and 13-14, respectively). All lanes were loaded with 15μg total RNA. The membrane was hybridised with ^{32}P-labelled CP-2 cDNA and exposed to X-Omat AR film for 48 hours (A). The membrane was stripped and reprobed with an oligonucleotide probe for the 18S ribosomal RNA to check that all lanes contained equivalent amounts of RNA (B). The membrane was stripped again and reprobed with a ^{32}P-labelled TP-2 cDNA to confirm loss of condensing spermatids due to MAA treatment (C).

At 3 days after MAA treatment, all stages of the spermatogenic cycle, with the exception of stages I and VIII-XI but including stages IV-VII when the mRNA for CP-2 is normally expressed, were depleted selectively of pachytene spermatocytes. An apparent increase in the amount of mRNA for CP-2 in total testicular RNA at this time after treatment was observed (see Fig.4). At 7 days after MAA treatment tubules at those stages at which CP-2 mRNA is normally expressed either lacked round spermatids (stages II-V) or had a full complement of germ cells (stages VI-VII). At this time point there was a small increase in CP-2 mRNA expression relative to intact controls (Fig.4). At 14 days after MAA treatment when round spermatids were depleted from tubules at all stages at which CP-2 is normally expressed (see Fig.5) a more marked increase in CP-2 mRNA expression on Northern blot was observed (Fig.4). In contrast, at 21 days after MAA treatment, when elongate spermatids from stages I-VI/VII of the cycle (including those stages at which CP-2 is usually expressed heavily; stages IV-VII) and round/elongating spermatids at stages VII-XII/XIII were depleted selectively (Fig.5) there was a major reduction in the signal for the 1.7kb transcript for CP-2 (Fig.4). Levels of this transcript had returned almost to control levels at 28 days after MAA treatment when elongate spermatids were depleted only from stages I-IV/V of the cycle and comparable results were obtained at 42 days after MAA treatment when all tubules had a full germ cell complement.

The comparative levels of CP-2 mRNA at each treatment time were calculated by densitometric measurements of the signal obtained by Northern blot. The effect of unequal gel loading was overcome by measuring the level of 18S rRNA (Fig.4b) and calculating the ratio of CP-2/18S RNA (Fig.6). The results obtained by scanning confirmed the original Northern blot analysis.

Maturation depletion of germ cells caused by MAA treatment was confirmed using a probe for transition protein 2 (TP-2) as a marker. TP-2 is a DNA binding protein expressed specifically in the testis. The mRNA for TP-2 has been localised in step 7 to step 13 spermatids during their elongation phase (Saunders *et al.*, 1992). When membranes were stripped and reprobed with a cDNA probe specific to TP-2 one abundant mRNA species of approximately 0.9kb was visualised (Fig.4c). The level of expression of this transcript remained constant in all MAA treated animals except at 14 and 21 days post treatment. At these time points TP-2 mRNA was significantly reduced or absent from the testis confirming the selective depletion/reduction of step 7-13 spermatids from the tubules at these times.

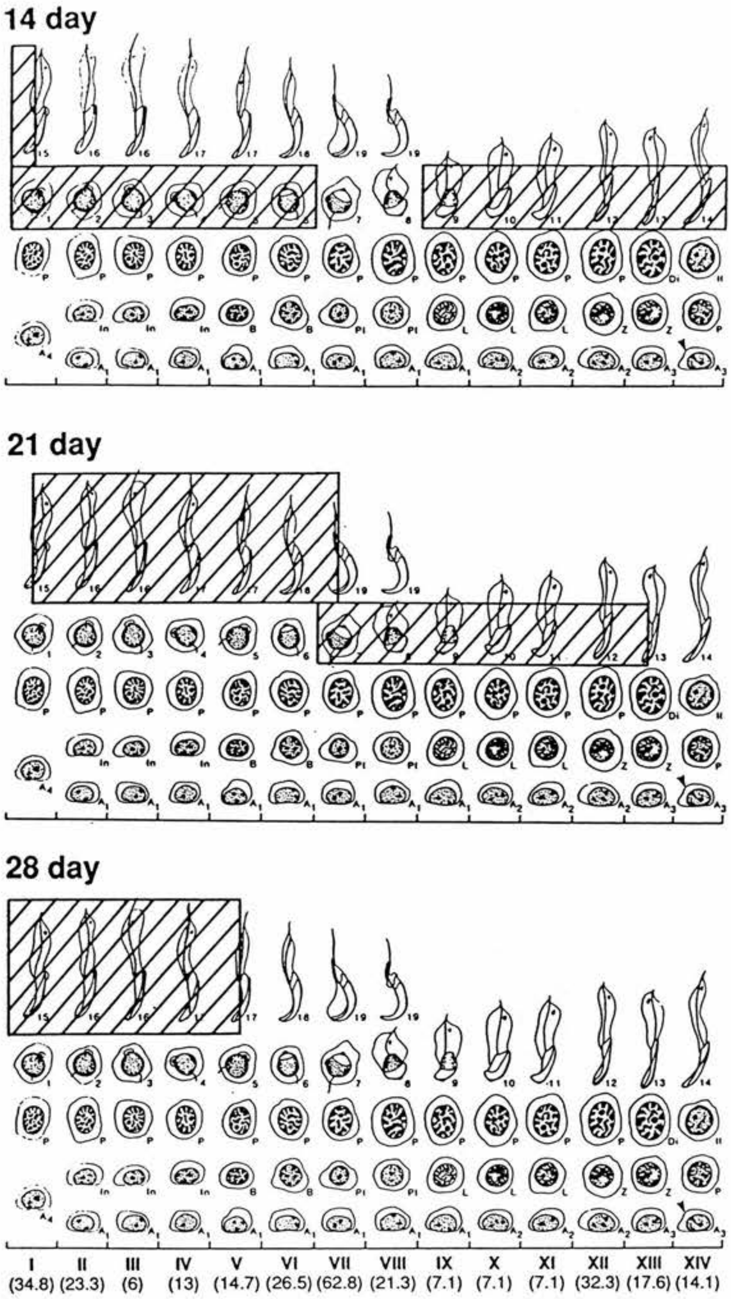


Figure 5. Germ cell depletion on MAA treatment. Pattern of depletion of germ cell types observed at 14, 21 and 28 days after administration of a single oral dose of 650mg/kg methoxyacetic acid (MAA). The stages (I-XIV) of the spermatogenic cycle and their duration in hours are shown at the bottom (based on Leblond and Clermont, 1952). MAA treatment initially causes a loss of 80-100% pachytene and later spermatocytes at all stages of the spermatogenic cycle with the exception of early to mid stage VII. As spermatogenesis progresses this initial lesion results in a selective loss of round and then elongate spermatids.

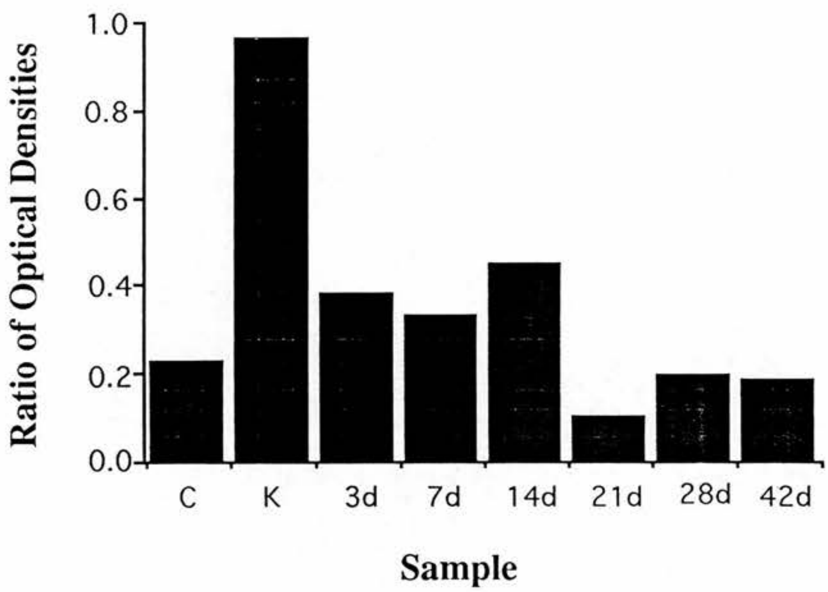
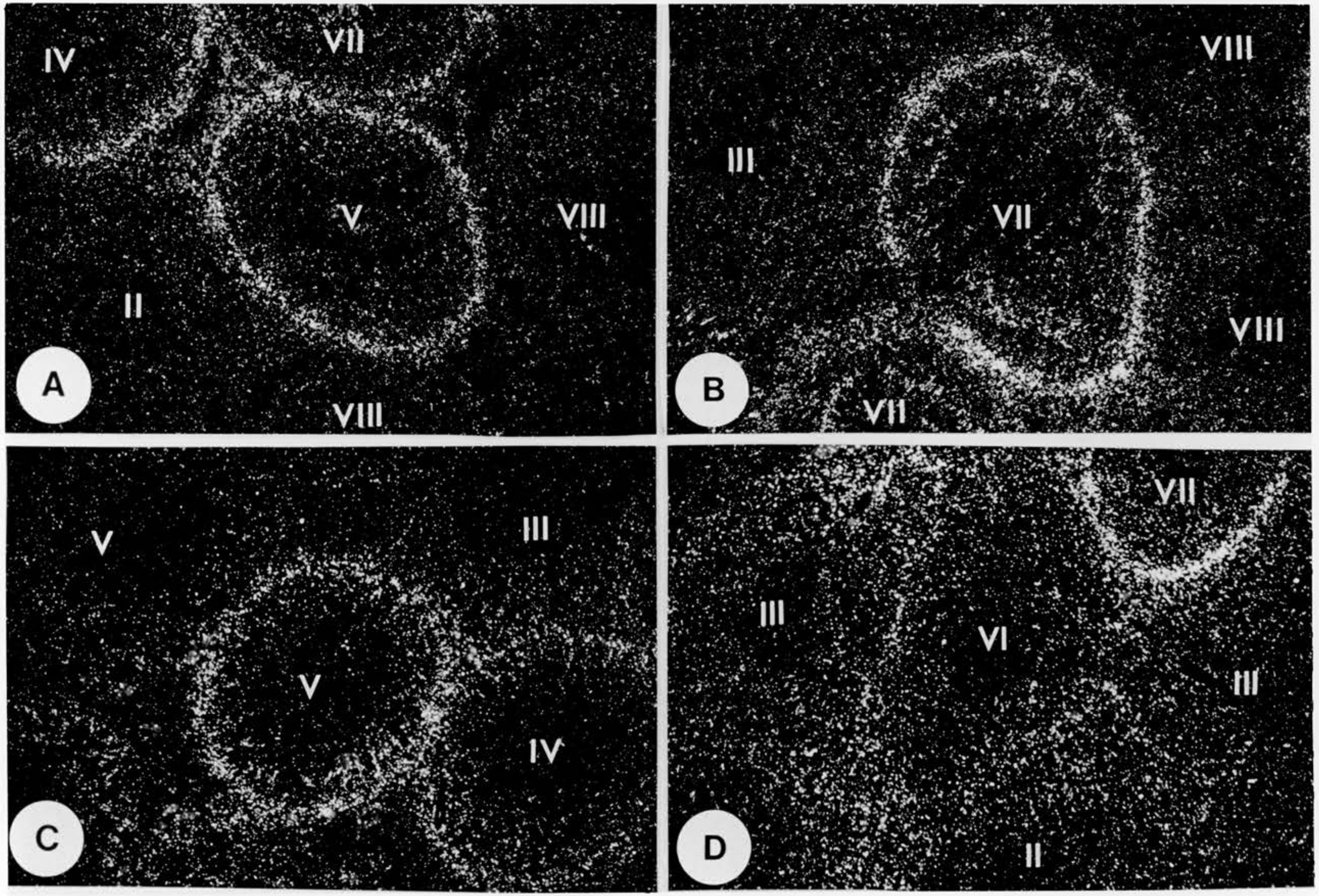


Figure 6. Effect of germ cell depletion on expression of CP-2 mRNA. Optical density of signals from panels A and B of Fig.4 were measured using image analysis. The ratios of CP-2 mRNA expression to 18S rRNA loading are plotted for each MAA time point to show the effect of treatment. C, control testis; K, kidney; 3, 7, 14, 21, 28 & 42, days after MAA treatment.

6.3.4 Germ cell influence on CP-2 production

Testicular sections from animals at selected times after MAA treatment were subjected to *in situ* hybridisation with a probe specific for CP-2 mRNA. In testes from control animals, CP-2 mRNA expression was observed in 37% of tubules. This value was obtained by counting 100 tubules at random from sections from each of two animals. At 3 days after MAA treatment, depletion of pachytene spermatocytes at those stages which normally express CP-2 mRNA did not appear to have any significant effect on the level of expression of the transcript (Fig.7b) or on the frequency of tubules expressing CP-2 mRNA (Table 1). At MAA + 7 days, pachytene spermatocytes at stages VIII-XIII and round spermatids at stages II-V were depleted from the seminiferous tubules. At this time point, tubules in which CP-2 mRNA is normally expressed, stages IV-V (missing round spermatids) and stages VI-VII (full germ cell complement), were still expressing mRNA for CP-2 (Table 1, Fig.7c). The depletion of round spermatids from tubules at stages when CP-2 is expressed (MAA + 14 days), except for stage VII which had a full complement of germ cells, did not lead to any change in the frequency of tubules showing a positive hybridisation signal (Table 1). In contrast, at 21 days after MAA treatment the amount of mRNA visualised by *in situ* hybridisation with the CP-2 riboprobe was significantly different from those of controls (see Figs.7d and 8). The frequency of seminiferous tubules showing a positive hybridisation signal was reduced (22% in MAA + 21 day compared to 37% in controls). Tubules at stages IV to early-midstage VII had been depleted of their elongate spermatids (step 17 to step 19 spermatids) and no longer expressed CP-2 mRNA. However, certain tubules at this time point did still express CP-2 mRNA. Morphological examination of these tubules revealed that both their round and their elongate spermatids were depleted (see Fig.8c); these tubules were thought to be at late stage VII of the spermatogenic cycle (see Ratnasooriya & Sharpe, 1989). It was also observed that certain tubules (thought to be stage VII) had elongate spermatids present in only part of the tubule. In these tubules CP-2 mRNA expression was seen only in those Sertoli cells with which elongate spermatids were still associated (see Fig.8d). At 28 days after MAA treatment, step 15 to step 17 spermatids were depleted from tubules at stages I-IV/V. CP-2 mRNA expression was not detectable in stage IV- early stage V while normal expression remained in late stage V- stage VII. There was no significant difference in the frequency of tubules expressing mRNA for CP-2 at this time point when compared to controls. At 42 days after treatment all tubules had a full complement of germ cells and there was no difference in the frequency or the level of expression at this time point when compared to controls.

Figure 7. Effect of germ cell depletion on CP-2 mRNA. Darkfield photomicrographs of sections probed with a ^{35}S-labelled riboprobe for CP-2. (A) Control section showing expression in a tubule at stage V with a full complement of germ cells. (B) Section from an animal at 7 days after MAA treatment when pachytene spermatocytes were depleted from the illustrated stage VII tubule. (C) 14 days after treatment with MAA showing a stage V tubule from which round spermatids were depleted. (D) Section from an animal at 21 days after treatment with MAA showing loss of expression in a stage VI tubule from which elongate spermatids had been depleted. Magnification x90.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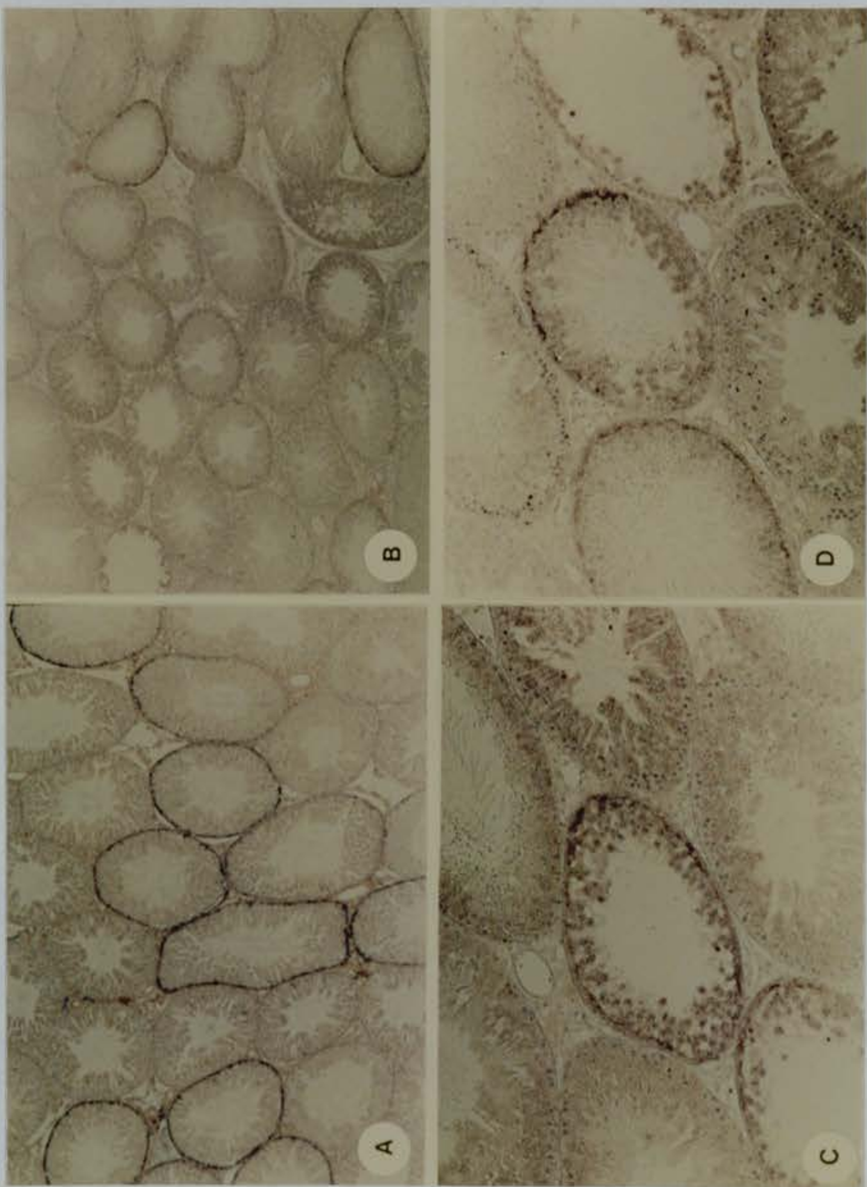

Figure 8. Loss of CP-2 mRNA expression on depletion of elongate spermatids. Brightfield photomicrographs of sections probed with a Dig-labelled riboprobe for CP-2. (A) Control testis showing stage specific expression. (B) Testis 21d after MAA treatment showing decrease in frequency of tubules expressing CP-2 mRNA compared to controls. Magnification x20. (C) Tubule at 21d after MAA treatment which is depleted of both round and elongate spermatids and is expressing CP-2 mRNA. (D) Stage VII tubule from the testis of a rat 21 days after MAA-treatment and in which only part of the epithelium was depleted of elongate (step 19) spermatids. Sertoli cells remaining associated with elongate spermatids show CP-2 mRNA expression while those with not associated elongate spermatids show no expression. Magnification x52.

Table 1. Effect of germ cell depletion on frequency of tubules expressing CP-2 mRNA.

Days after MAA treatment	Percentage tubules expressing
Control	37%
3d	33%
7d	37%
14d	36%
21d	**22%**
28d	30%
42d	33%

Frequency of tubules expressing CP-2 mRNA at specific times after MAA treatment, determined by counting approximately 100 randomly picked tubules from each of 2 rats for each time point.

Immunostaining using the antibody for CP-2 was carried out on testis sections from animals at selected time points after MAA treatment as for the mRNA studies. These results extended and confirmed those obtained by *in situ* hybridisation. At 21 days after MAA treatment, the frequency of tubules staining positively for CP-2 was decreased compared to that of control animals (Figs.9a & 9b) and certain tubules had positive staining for CP-2 only in part of their epithelium (stage VII; results not shown). There was a positive correlation between the presence of elongate spermatids and the presence of CP-2 in the Sertoli cell cytoplasm. This result was shown to be specific for CP-2 since when an antibody for SGP-1 was used there was no obvious decrease in Sertoli cell SGP-1 content at 21 days after MAA treatment compared to controls (Figs.9c & 9d).

6.4. Discussion

CP-2 mRNA was localised to the cytoplasm of the Sertoli cells of the seminiferous epithelium by use of non-radioactive *in situ* hybridisation. Expression of the CP-2 mRNA was shown to be stage specific; the mRNA was expressed only in tubules from stage IV to stage VII of the spermatogenic cycle. A similar pattern of expression has been shown by Northern analysis of isolated tubules at different stages of the spermatogenic cycle (Erickson-Lawrence *et al.*, 1991). These authors observed maximal expression of CP-2 mRNA at stages VI and VII, decreased expression at stage VIII and no detectable expression at stages XII and II. Immunostaining showed

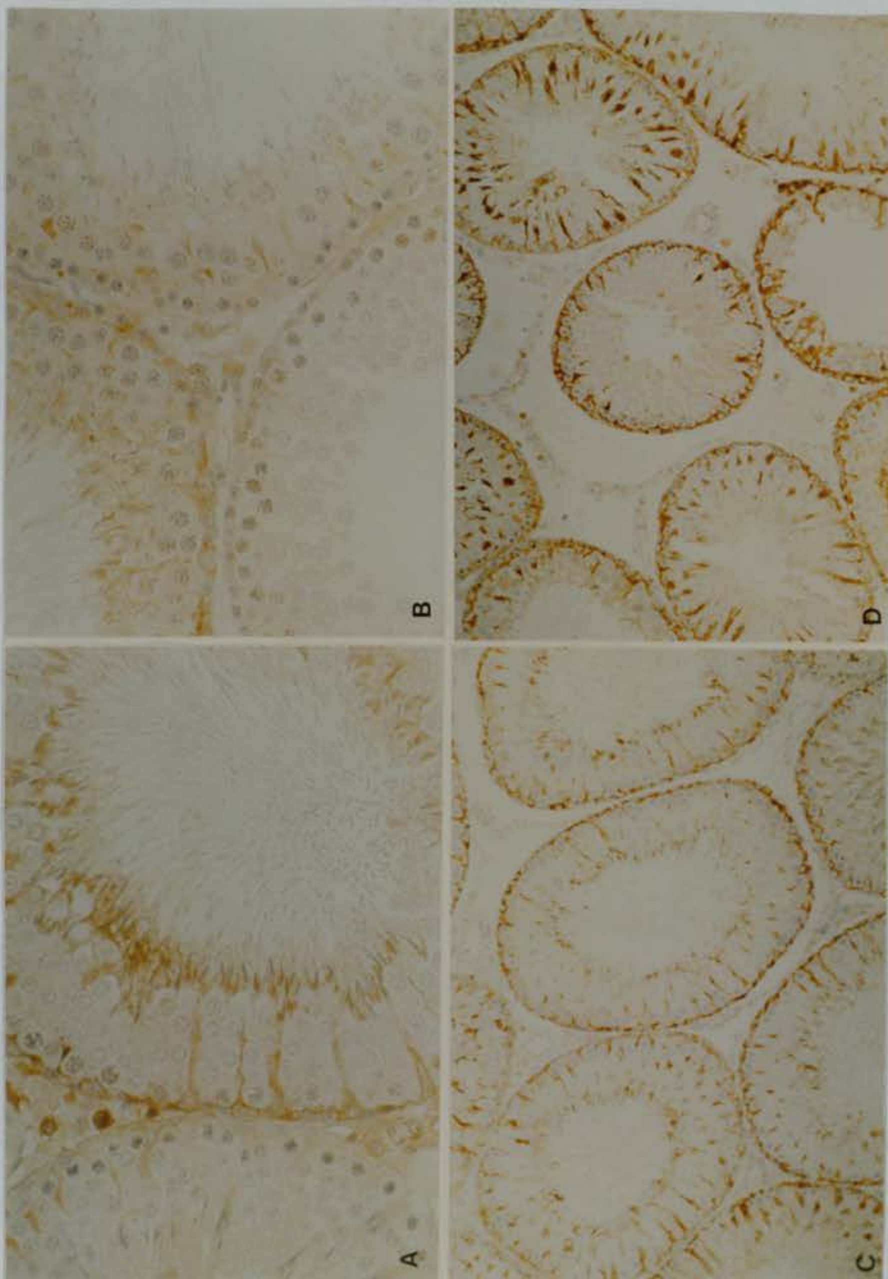

Figure 9. Effect of elongate spermatid depletion on CP-2 and SGP-1 production. Brightfield photomicrographs of cross sections of perfusion fixed rat testis. The tissue was incubated with anti-rat CP-2 (A & B) or anti-rat SGP-1 (C & D) as primary antibody. (A & C) Control rat testis showing localisation and frequency of tubules containing CP-2 and SGP-1 protein. (B & D) Sections 21d after MAA treatment showing decrease in frequency of tubules containing CP-2 but not SGP-1 when elongate spermatids are depleted from the seminiferous epithelium. x129 (A & B) and x52 (C & D) magnifications.

that Sertoli cell CP-2 protein content reflected the mRNA expression with stage specific synthesis only at stages V-VIII of the spermatogenic cycle.

The effect of testicular maturation on CP-2 production has previously been studied (Zabludoff *et al.*, 1990b). The synthesis and secretion of CP-2 by isolated seminiferous tubules was shown to increase significantly between 17 and 75 days of age with the greatest increase occurring between 35-45 days. Immunostaining detected CP-2 in Sertoli cells at stages VI and VII of the spermatogenic cycle of 36 day old rats with this stage specific synthesis increasing significantly by day 38. This did not correspond to the appearance of any new germ cell types in the testis as the most mature germ cell at stages VI-VII at both these ages was round spermatids. These authors did not detect CP-2 in animals younger than 36 days old. However, the present study found expression of CP-2 mRNA as early as postnatal day 16. The most mature germ cell type in the testis of 16 day old animals are preleptotene spermatocytes (Clermont & Perry, 1957) and staging of the individual tubules according to the classification system of Leblond and Clermont (1952) is obviously not possible. However, expression of CP-2 mRNA did not occur in all tubules indicating that some stage specificity had already been established. A much more pronounced stage specific expression was visible in the testis of 23 day old rats in which the most advanced germ cell type was step 1 spermatids. By 32 days of age step 12 elongating spermatids are present in some tubules while in 42 day old rats the first wave of spermatogenesis has almost been completed. Stage specific expression of CP-2 mRNA in testis from rats of these ages is well established. These results show stage specific expression of CP-2 mRNA in animals well before synthesis and secretion of the protein has been shown. This suggests that transcription of CP-2 occurs as early as postnatal day 16 but that the mRNA is not translated until day 35, or the difference in the timing of mRNA expression and protein synthesis could be due to a lack of sensitivity of the protein detection system. Despite these differences the establishment of a cycle of expression of CP-2 in animals at an age when the first wave of spermatogenesis has not been completed has been shown. These data could be consistent with the pattern of expression being an integral function of the Sertoli cell rather than being regulated by the presence of germ cells as has previously been thought. However, it has been shown that the increase in secretion of CP-2 between the immature and the adult rat does not occur when germ cells are not present (Wright *et al.*, 1989). Sertoli cells were isolated from 20 day and 65 day old rats and placed in culture for 6 days. Incorporation of radiolabelled methionine into newly synthesised CP-2 was measured and there was no increase in secretion observed by Sertoli cells from the older rats in contrast to what had been demonstrated using seminiferous tubules. It has been proposed that as the Sertoli cell matures control of its function is taken over by the more advanced germ cell types as they appear in the seminiferous epithelium (Jégou *et al.*, 1992). Therefore, germ cells which are important in the control of CP-2 stage specific expression in the immature animal may not be important in the adult (see later discussion).

The major finding of the study outlined in this chapter was the modulation of CP-2 mRNA levels by elongate spermatids. CP-2 mRNA expression was shown both by Northern blot and *in situ* analysis to be reduced in testes recovered from animals at 21 days after treatment with MAA compared with controls. At this time point, seminiferous tubules at most stages of the spermatogenic cycle, with the exception of stages XIII-XIV and late stage VII to stage VIII, were depleted of elongating (steps 8-12) or elongated (steps 15-18) spermatids. At 21 days after MAA treatment, tubules at stages IV-early stage VII at which there would normally be expression of CP-2 mRNA did not show a positive signal when examined by *in situ* hybridisation. These tubules were depleted of elongate spermatids but retained their spermatogonia, pachytene spermatocytes and round spermatids. The results are therefore consistent with the full expression of CP-2 mRNA being dependent upon the presence of elongate spermatids. It was also shown by immunostaining using an antibody specific for CP-2 that there was a decrease in the number of Sertoli cells positively staining for CP-2 protein at 21 days after MAA treatment. This demonstrates that the modulation of CP-2 mRNA expression by elongate spermatids results in a decrease in the level of CP-2 protein in the seminiferous epithelium.

In contrast to these results, at 28 days after MAA treatment no significant change in the level of mRNA for CP-2 was observed, although elongate spermatids were depleted from tubules at stages I-IV and early stage V of the spermatogenic cycle. The frequency of occurrence of tubules at stages IV (4.8%) and V (6.8%) in the testis is very low compared to that of stage VII (20.9%; Hess *et al.*, 1990). Therefore the effect of depletion at this point is not as apparent on Northern analysis of total testicular RNA as at 21 days after MAA treatment when stages IV to early- to mid-stage VII were all depleted of elongate spermatids. *In situ* hybridisation of tissue recovered 28 days after MAA treatment did confirm the loss of CP-2 mRNA expression in tubules at stages IV and V which were lacking elongate spermatids. This demonstrates the importance of carrying out *in situ* hybridisation in conjunction with Northern analysis studies as in this case the former technique has been more informative.

This study has also shown that at 21 days after MAA treatment the expression of mRNA for CP-2 was maintained in some tubules which were depleted of elongate spermatids. Using brightfield microscopy, it was determined that these tubules were depleted not only of elongate spermatids but also of round spermatids. These tubules were determined to be at late stage VII based on a number of criteria such as the presence of preleptotene spermatocytes and the pattern of germ cell depletion calculated to occur at this time (Bartlett *et al.*, 1988; Ratnasooriya & Sharpe, 1989). Therefore, it is possible that round spermatids may also be involved in modulating Sertoli cell expression of CP-2 mRNA at certain stages of the spermatogenic cycle. In support of this idea Northern blot analysis showed an apparent increase in the amount of CP-2 mRNA in testes recovered from animals 7 and 14 days after MAA treatment, times at which round spermatids were depleted from tubules at most of the stages (IV-VII) at which CP-2 mRNA is normally expressed. The frequency of tubules expressing CP-2 mRNA at these time points, as observed by *in situ* hybridisation, did not differ from that of controls. It is possible that the level of CP-2 mRNA expressed at stages IV-VII of the spermatogenic cycle in sections from animals at 7 and 14 days after MAA treatment is increased compared to controls. Unfortunately, quantification using the available autoradiography software packages for image analysis has proved impossible due to the discrete localisation of the signal around the base of the seminiferous tubules. However, it has been shown by 2D-gel electrophoresis that depletion of round spermatids due to MAA treatment causes an apparent increase in secretion of CP-2 protein by isolated seminiferous tubules at stages VI-VIII compared to that secreted by tubules at the same stages isolated from control animals (Sharpe *et al.*, 1993a). These observations lead to the speculation that CP-2 production may also be regulated (negatively) by round spermatids at the level of transcription in the adult seminiferous tubule.

It has been shown previously that CP-2 secretion by seminiferous tubules increases in the developing testes as the number of round spermatids increases (Zabludoff *et al.*, 1990b). This may indicate that in contrast to the findings of the present study, round spermatids stimulate rather than inhibit CP-2 production during pubertal growth of the rat testis. However, evidence has been presented in support of the idea that the response of the Sertoli cell to germ cells is dependent on the stage of maturation of the Sertoli cells. For example, it has been shown that round spermatids cause an increase in the levels of α-inhibin mRNA when cultured with Sertoli cells isolated from 20-day old rats (Pineau *et al.*, 1990). In contrast, secretion of α-inhibin in vivo by Sertoli cells in adult rats decreased only in the absence of elongate spermatids while it was unaffected by depletion of round spermatids (Allenby *et al.*, 1991). It has been hypothesised that modulation of Sertoli cell function during development of the testis is transferred sequentially to the most advanced generation of germ cells present within the seminiferous epithelium (Jégou, 1991). A similar situation may apply to the regulation of CP-2 production in the developing rat testis compared to that found in the mature animal.

Several studies have implicated spermatids in the modulation of various aspects of Sertoli cell function as described in previous chapters. Addition of round spermatids to immature Sertoli cells in culture has been shown to cause an increase in the secretion of androgen binding protein (ABP; Le Magueresse *et al.*, 1986) as well as α-inhibin (Pineau *et al.*, 1990). Maturation depletion of germ cells, initiated by γ-irradiation of the testis, showed a strong correlation between ABP production and the presence of elongate spermatids (Pineau *et al.*, 1989) while depletion of elongate spermatids from the tubules following MAA treatment led to a decrease in secretion of immunoreactive α-inhibin *in vivo* and *in vitro* as previously stated (Allenby *et al.*, 1991). Elongate spermatids have also been reported to play a role in regulating the production of seminiferous tubule fluid by the Sertoli cell (Jégou *et al.*, 1984), the volume of the lumen of the seminiferous tubule (Sharpe, 1989) and overall protein secretion (McKinnell & Sharpe, 1992). The modulation of CP-2 mRNA expression (and ultimately production of the protein) by elongate spermatids, and possibly also by early spermatids as shown here, is therefore yet another example of germ cell modulation of Sertoli cell function.

Northern blot analysis also indicated an apparent increase in CP-2 mRNA levels at 3 days after MAA treatment. At this time point, tubules at all stages of the spermatogenic cycle, with the exception of stages I and VIII-XI, were depleted of most pachytene spermatocytes. It is well established that pachytene spermatocytes are very active transcriptionally, and contain larger amounts of mRNA than do other testicular cell types (Monesi *et al.*, 1978). It is therefore possible that depletion of the majority of pachytene spermatocytes at this time point leads to a much higher percentage of Sertoli cell RNA being loaded in lanes from rats at MAA+3 days compared to that in the control lanes, as equal amounts of total RNA were loaded onto each lane of the denaturing agarose gel. This interpretation is supported by the absence of any obvious change at 3 days after MAA treatment in the mRNA level for CP-2 when assessed by *in situ* hybridisation. Furthermore, the absence of pachytene spermatocytes has no effect on the secretion of CP-2 protein by isolated seminiferous tubules (C. McKinnell & R.M. Sharpe, unpublished data). However, the possibility that pachytene spermatocytes also exert a modulatory effect on CP-2 gene expression cannot be ruled out completely.

CP-2 has been identified from sequence data as the proenzyme form of cathepsin L, a cysteine protease. A known function of proteases is their involvement in facilitating tumour invasion and metastases by degrading the components of extracellular membranes and it has been reported that cathepsin L is expressed at higher levels in cancers than in normal tissues (Chauhan *et al.*, 1991). The proenzyme form of cathepsin L has a MW of approximately 39K. This form has been shown to be activated in the acidic milieu of lysosomes and in the environment created by tumour cells on tissue invasion (Gal & Gottesman, 1986). Immunohistochemical analysis of CP-2 expression showed a localisation of the protein in the microenvironment between the elongating spermatids and the Sertoli cells (the present study; Zabludoff *et al.*, 1990a). CP-2 may be active here since the secretion of lactic acid by the Sertoli cell gives them the potential for acidifying this microenvironment (Oonk *et al.*, 1989). It is therefore possible that the expression of cathepsin L/CP-2 in the testis leads to a limited movement of germ cells within the seminiferous epithelium in a similar manner to that found in tumour tissue.

Substrates for the catalytic activity of cathepsin L have been identified in various tissues; they include the extracellular matrix proteins fibronectin, collagen and laminin, as well as elastase and α1-proteinase inhibitor (Kirschke *et al.*, 1982; Gal & Gottesman, 1986; Johnson *et al.*, 1986; Mason *et al.*, 1986). It has been proposed that the protease activity of CP-2 is involved in the breakdown of interactions between spermatids and Sertoli cells as suggested above (Erickson-Lawrence *et al.* 1991). Elongate spermatids are released into the lumen of the seminiferous tubule towards the end of stage VIII of the spermatogenic cycle. This release is preceded by a movement of the elongate spermatids in the epithelium from the base of the Sertoli cell at stages IV/V to the apical region at stages VI-VIII. There are several specialised forms of intercellular adhesions between Sertoli cells and spermatids which are unique to the testis (reviewed by Russell, 1980; 1993a). Ectoplasmic specialisations are formed between Sertoli cells and elongating spermatids in the adluminal region of the seminiferous epithelium and are thought to anchor elongating spermatids deep into invaginations of Sertoli cell plasma membranes (Russell, 1977). As spermatids move towards the lumen of the seminiferous tubules ectoplasmic specialisations begin to disappear; it is thought this is achieved by endocytosis of the junctional connections leading to loss of adhesion between the Sertoli cell and the spermatids (Russell, 1991). These adhesions have been implicated in movement of the spermatids within the tubules and release of the fully mature spermatids from the testis with the control of these processes residing in the Sertoli cell. The data presented in this chapter are consistent with control of this movement being initiated by the elongate spermatids themselves, as opposed to control residing with the Sertoli cells as was previously believed, although the mechanism by which this modulation occurs is as yet unclear.

In conclusion, the present study has shown that expression of CP-2/cathepsin L mRNA by Sertoli cells is modulated by spermatids, in particular by elongate spermatids at stages IV-VII of the spermatogenic cycle. The mechanism by which the spermatids control expression has not been identified. It is proposed that the spermatids may regulate their own movement within and release from the seminiferous epithelium by modulating transcription of the CP-2 gene and hence the production of CP-2 by Sertoli cells.

7. The use of subtractive hybridisation to identify novel Sertoli cell mRNAs modulated by elongate spermatids

7.1. Introduction

The preceding chapters of this thesis have presented results from investigations into the regulation of genes known to be expressed by Sertoli cells. The data has suggested that germ cells have a role to play in the regulation of specific mRNA expression in Sertoli cells. Chapter 6 specifically demonstrated an influence of elongate spermatids on expression of CP-2 mRNA and ultimately its protein product. Other investigators have shown that spermatids may be important regulators of Sertoli cell function. For example, the production of seminiferous tubule fluid and the size of the tubule lumen are positively influenced by late spermatids (Sharpe, 1989; Jégou *et al.*, 1984). It has also been demonstrated that depletion of elongate spermatids results in a decrease in the secretion of immunoreactive inhibin and ABP by the Sertoli cell (Allenby *et al.*, 1991; Pinon-Lataillade *et al.*, 1988). Several other studies have provided data consistent with the importance of elongate spermatids (for review see Jégou *et al.*, 1992) and this growing awareness of the involvement of these germ cells in the regulation of spermatogenesis led to the study reported in this chapter. This investigation employed the technique of subtractive hybridisation to attempt to identify Sertoli cell mRNAs whose expression may be positively regulated by elongate spermatids.

Subtractive hybridisation is a technique whereby cDNA sequences common to normal and experimentally or naturally altered states are removed and only those sequences unique to one state are retained. This technique has been used successfully to identify differentially regulated genes in a number of systems (Hedrick *et al.*, 1984; Duguid *et al.*, 1988). In this study I attempted to identify genes which are expressed by Sertoli cells only when elongate spermatids are present in the seminiferous epithelium of the rat testis. The technique (strategy shown in Fig.1) involved the synthesis of cDNAs from Sertoli cell mRNA isolated from rats 21 days after MAA treatment when most tubules are depleted of elongate spermatids. These were "subtracted" from cDNAs synthesised from normal rat testis mRNA in the assumption that the sequences remaining should be dependent on the presence of elongate spermatids for their expression. These "subtracted" cDNAs were then used to screen a cDNA library prepared from mRNA isolated from a testis cell fraction enriched for Sertoli cells to enhance the chances of selecting for genes expressed in Sertoli cells. The data

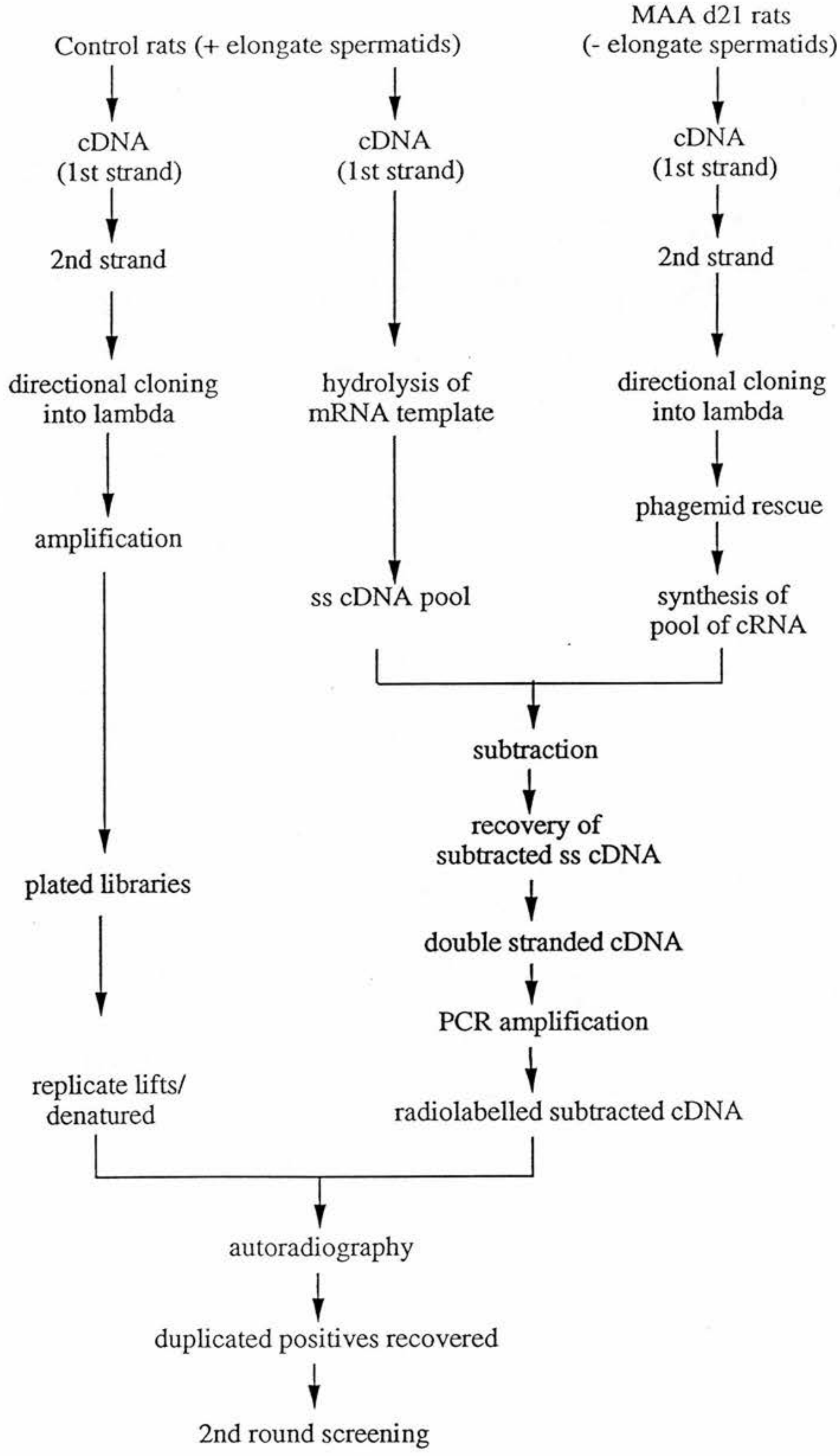

Figure 1. Subtractive hybridisation strategy. Flow chart outlining the main steps performed in the technique of subtractive hybridisation.

presented in this chapter describes the methodology used to attempt the isolation of novel elongate spermatid regulated Sertoli cell mRNAs and some preliminary results.

7.2. Experimental Procedures

7.2.1 Sertoli cell isolation

Adult rats were treated with methoxyacetic acid as described in chapter 3. Twenty one days after treatment rats were killed, the testis removed and Sertoli cells isolated. Cells were also isolated in an identical way from age matched control (untreated) rats. The isolation procedure was as described in chapter 5.

Cells (approximately 5 x $10^{4)}$ from fraction 4 in a volume of 100µl were fixed onto a TESPA coated slide by centrifugation at 800g for 5min using a cytospin (Shandon Elliott) The slides were air dried briefly, fixed in Bouins' fixative (see chapter 3) for 2min, washed in distilled water and air dried. Immunocytochemistry using an antibody to the Sertoli cell protein, SGP-1 was carried out to determine the purity of the enriched Sertoli cell fraction. Immunostaining was carried out as described in chapter 3, section 11 with SGP-1 antibody used at a dilution of 1:1000.

7.2.2 Preparation of Sertoli cell mRNA and cDNA synthesis

Fraction 4 from the elutriation procedure was chosen to isolate Sertoli cell RNA. This fraction contained mainly Sertoli cells with some late spermatid contamination. Pelleted cells were resuspended in 2ml of solution D (chapter 3) and were passed through a 16 gauge needle to rupture the cells and shear some of the DNA. Preparation of total RNA was continued as described in chapter 3. Messenger RNA was isolated from Sertoli cell total RNA by fractionation on a sterile minicolumn containing oligo dT cellulose. Briefly, oligo dT cellulose (0.5ml; Pharmacia) was suspended in loading buffer B containing 20mM Tris, pH7.4, 0.1M NaCl and 1mM EDTA and allowed to expand overnight at 4°C. The slurry was poured into a disposable plastic column and allowed to settle. The column was washed with 3ml 0.1M NaOH containing 5mM EDTA followed by water until the pH of the effluent was less than 8.0. Finally, it was equilibrated with loading buffer A which contained 40mM Tris, pH7.4, 1M NaCl and 1mM EDTA.

Sertoli cell total RNA (470µg for control RNA and 400µg for MAA + 21d RNA) was dissolved in 500µl pure water and heated to 65°C for 5min. An equal volume of prewarmed loading buffer A was added and the mix allowed to cool to room temperature for 2min before adding to the column. The eluate was collected, heated to 65°C for 5min and reapplied to the column. This was washed with 5ml of loading buffer B and the eluate containing non-poly (A^+) RNA was discarded. Elution buffer

(1.5ml) containing 10mM Tris, pH7.4 and 1mM EDTA was added and fractions of 10 drops each were collected. RNA containing fractions were identified using an ethidium bromide dot blot (see section 3.5.3), the fractions pooled and precipitated on dry ice for 30min with 1/10th volume of 3M sodium acetate, pH5.5 and 2.5 volumes of ethanol. RNA was pelleted by centrifugation (13,000rpm for 30min at 4°C), washed with 80% ethanol, dried and resuspended in pure water.

Synthesis of cDNA and preparation of the Sertoli cell cDNA libraries was carried out using the Stratagene Zap-cDNA synthesis kit and following the manufacturers instructions based on the method of Okayama & Berg (1982; see Fig.2). The first strand of cDNA was synthesised using a poly dT primer linked to a Xho I restriction enzyme recognition site and Sertoli cell mRNA as a template. The poly dT region binds to the poly A tail of the mRNA template and the cDNA was synthesised using reverse transcriptase. Briefly, 5μg poly (A+) mRNA was allowed to anneal to the linker-primer at room temperature for 10min. The reaction mixture contained DTT, RNase Block II to prevent hydrolysis of the mRNA template, dATP, dGTP and dTTP, and 5-methyl dCTP. This dCTP analogue was used to protect the cDNA from restriction enzyme digestion in subsequent cloning steps. After annealing was complete reverse transcriptase was added and the reaction was incubated at 37°C for 1h. Synthesis of the first strand of cDNA was complete at this point.

Second strand synthesis was carried out in the presence of RNase H and DNA Polymerase I. RNase H nicks the RNA bound to the first strand cDNA and these fragments then served as primers for DNA polymerase I in the second strand synthesis. The second strand reaction mixture contained dideoxynucleotides with an excess of dCTP. This reduced the possibility of 5-methyl dCTP becoming incorporated into the second strand and therefore ensured that the linker-primer formed at the ends of the DNA will be susceptible to restriction enzyme digestion. The reaction mix also contained 20μCi α-^{32}P dCTP (800Ci/mmol) to allow analysis of the quality and quantity of cDNA synthesised. Synthesis of second strand cDNA was carried out at 16°C for 2.5h. After synthesis, enzymes were removed by phenol:chloroform extraction, the cDNA precipitated with 3M sodium acetate and absolute ethanol at -20°C overnight and resuspended in sterile water.

7.2.3 Preparation of cDNA libraries

The termini of the cDNA were blunted by incubation with T4 DNA polymerase. This reaction was carried out at 37°C for exactly 30min and the blunted cDNA was phenol:chloroform extracted, precipitated, resuspended in ligation reaction buffer (Stratagene) and ligated to adaptors which had Eco RI cohesive ends. The ligation was

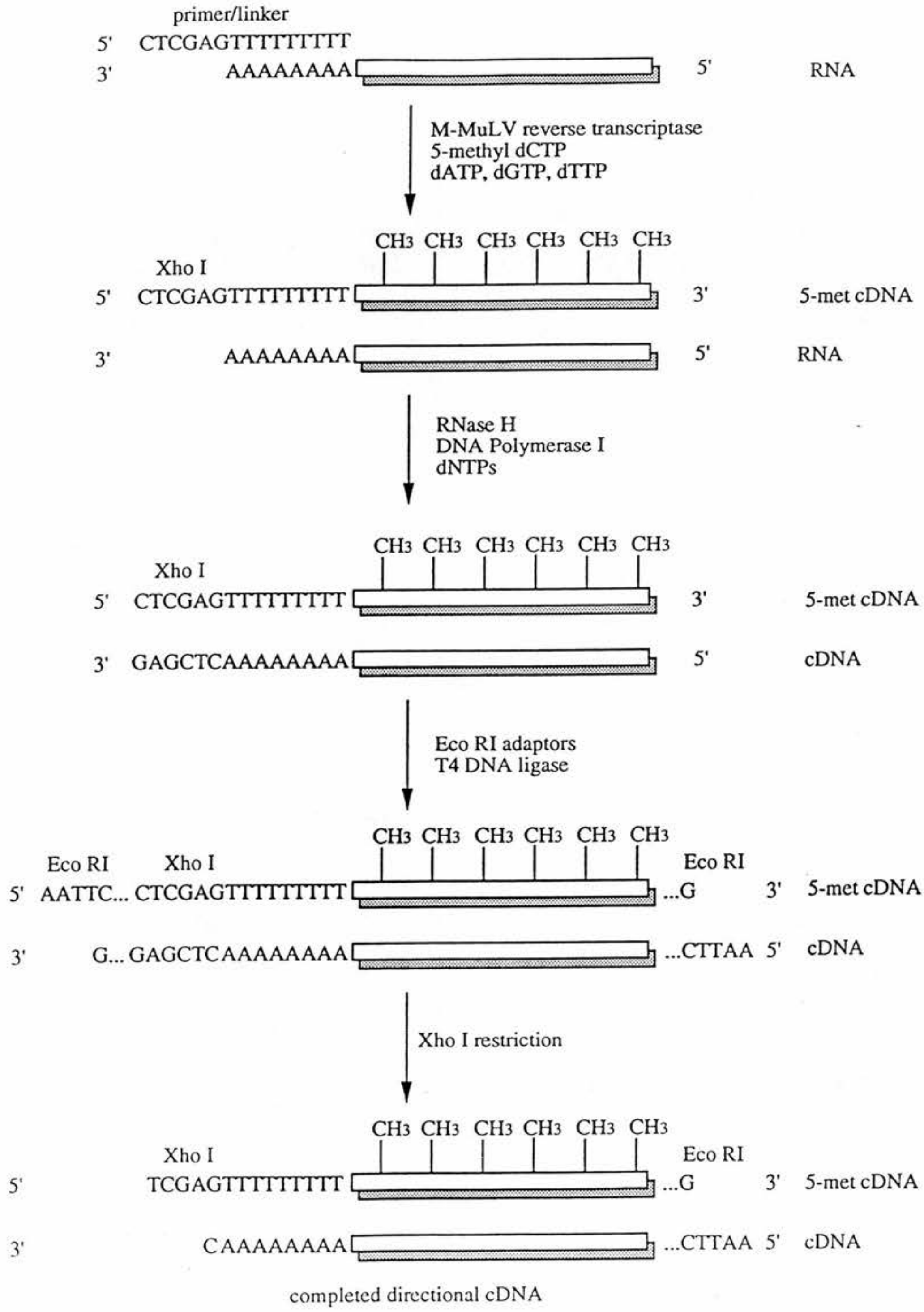

Figure 2. Outline of cDNA synthesis. Schematic of protocol used to synthesise directional cDNA from mRNA isolated from an enriched fraction of Sertoli cells. *Adapted from instruction booklet for the Zap-cDNA synthesis kit (Stratagene).*

carried out using 4U T4 DNA ligase at 8°C overnight after which the ligase was inactivated by heating at 70°C for 30min. The adaptor ends were phosphorylated with ATP in the presence of 10U T4 polynucleotide kinase at 37°C for 30min and the enzyme was heat inactivated as before. The cDNA was finally digested with 120U Xho I at 37°C for 1.5h

Each of the cDNA pools (control and MAA) was size selected by passing through a Sephacryl S-400 column. The column was equilibrated with 1x STE (contains 150mM NaCl, 2.5mM Tris and 0.25mM EDTA), the cDNA loaded and the first fraction collected by centrifugation at 600g for 5min. STE (60µl) was added to the column and this was centrifuged as before to collect fraction two. This was repeated once more for collection of a third cDNA fraction. Samples (5µl) were removed from each fraction to assess the efficiency of size selection and the remainder of the cDNA was phenol:chloroform extracted, precipitated overnight at -20°C and resuspended in sterile water. The pooled cDNAs (100ng) were ligated to 1µg Uni-ZAP XR vector in the presence of 1 x ligation buffer and 1mM rATP. The reaction was catalysed by 2U T4 DNA ligase at 12°C for 16-20h.

Samples of the S400 eluates were analysed by separation on a non-denaturing 5% (w/v) mini polyacrylamide gel prepared by combining 8.1ml 40% acrylamide, 10.5g urea, 5.7ml water and 2.5ml 10 x TBE (appendix I). This was polymerised with the addition of 100µl 10% ammonium persulphate and 20µl TEMED. The gel was poured between glass plates using a syringe, the comb inserted and the gel allowed to polymerise. Gel loading buffer containing 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF and 15% w/v Ficoll was added to 5µl cDNA sample and this was loaded onto the gel with DNA markers. The gel was submerged in 1 x TBE buffer and DNA electrophoresed at 100V for approximately 1.5h in a BioRad 'Mini PROTEAN II' electrophoresis cell.. The gel was stained in a solution of ethidium bromide in 1 x TBE and DNA visualised under UV light.

7.2.4 Packaging, titering and amplification of library

Ligation mix (1µl) was packaged into phage using the Gigapack II Gold packaging extract (Stratagene) according to the manufacturers instructions. Briefly, ligated cDNA/lambda was added to the packaging extracts on ice and incubated at room temperature for 2h. SM buffer (500µl; appendix I) and 20µl chloroform was added and the titre of recombinant phage determined as below.

SURE cells were obtained as a glycerol stock from Stratagene. These were streaked out onto LB agar plates containing 12.5µg/ml tetracycline and colonies allowed to form overnight at 37°C. A liquid culture was set up in LB media (appendix

I) containing 0.2% maltose and 10mM $MgSO_4$ and was inoculated with a single colony from the agar plate. Cells were grown to log phase with shaking at 30°C overnight, centrifuged at 1600g for 10min and resuspended in half their original volume of 10mM $MgSO_4$. NZY agar plates (appendix I) were poured and NZY top agar (appendix I) was melted and incubated at 48°C.

The packaged recombinant phage library was diluted 1:10, 1:100 and 1:1000 in SM buffer. Undiluted phage (1μl) and 1μl of each dilution was added to 200μl of previously prepared plating cells and the phage were allowed to attach at 37°C for 15min. Top agar (3ml) was added and the cell suspension was plated onto prewarmed NZY plates (37°C). The agar was left to harden and the plates incubated inverted at 37°C overnight. Titres of the libraries were determined by counting the number of plaques formed on the agar plates.

SURE cells for plating were prepared as described above. The primary library was amplified by combining 5 x 10^4 aliquots of packaged lambda clones with 600μl SURE cells and incubating at 37°C for 15min (8 plates for control library; 6 plates for MAA library). NZY top agar (6.5ml) at 48°C was added and the cells plated onto 150mm NZY agar plates. These were incubated at 37°C for approximately 7h. SM buffer (10ml) was added to the plates and this was incubated at 4°C overnight with gentle shaking. The SM buffer containing bacteriophage was removed from the plates. These were washed with an extra 2ml SM buffer and the suspensions were pooled. Cell debris was removed by centrifugation at 4000g for 5min, chloroform was added to the supernatant to 0.3% and the amplified library was stored in aliquots at 4°C. The titres of the amplified libraries were determined as before.

7.2.5 Phagemid rescue and transcription of RNA

The subtractive hybridisation technique requires approximately ten times as much Sertoli cell mRNA from the MAA treated animals as from the control animals. The aim of the large scale phagemid rescue from the unidirectional amplified MAA + 21d cDNA library was to provide templates for RNA transcription. Therefore, large amounts of mRNA can be produced without the necessity of sacrificing many rats.

Phagemid rescue. A large scale excision of recombinant Bluescript phagemids from the amplified MAA + 21d cDNA library was carried out according to Stratagene instructions as follows. Overnight cultures of XL1-blue and SOLR cells were set up in LB-broth containing 0.2% maltose and 10mM $MgSO_4$. The cells were centrifuged at 1600g for 10min and resuspended in 10mM $MgSO_4$ to an $O.D._{600}$ of 1.0. Aliquots of the library (5μl or 10μl, approximately 2 x 10^7pfus and 4 x 10^7pfus, respectively) were combined with 2 or 4 x 10^8 XL1-blue cells and 20-40μl ExAssist helper phage (>

1×10^7 pfu/ml) and incubated at 37°C for 15min before 20ml LB-broth was added and the tubes incubated at 37°C with shaking for 3h. During this time in the presence of the helper phage the phagemid was rescued from the lambda vector and secreted from the E.coli. Bacterial cells were removed from the supernatant by heating at 70°C for 20min, the cell debris centrifuged at 2500g for 10min and the supernatant retained.

Rescued phagemid was transfected into fresh E.coli cells. Several tubes containing aliquots of supernatant (10μl in each) were mixed with 500μl SOLR cells and incubated at 37°C for 15min. An aliquot of the cell suspension (250μl) was spread onto 150mm LB-agar plates containing 50μg/ml ampicillin and incubated at 37°C overnight to allow growth of colonies. LB-broth (5ml) was added to the plates and these were shaken for 1h at room temperature to slough off the colonies. The broth was taken off the plates, glycerol was added to 15% and these stocks of pooled phagemids containing a mixture of cDNAs generated from RNA of MAA + 21d rats were stored at -20°C.

Preparation of RNA from MAA library. Glycerol stocks (50μl) of the MAA+21d Sertoli cell library were used to inoculate 50ml LB-broth containing 50μg/ml ampicillin and 0.2% glucose and cultures were grown with shaking overnight at 37°C. Cells were centrifuged at 1600g for 10min and plasmid DNA extracted using the Promega 'Magic Maxiprep' kit following the procedure outlined for minipreps in chapter 3. Plasmid DNA was linearised with Bst XI in reaction conditions described previously (see chapter 3). The linearised DNA was phenol:chloroform extracted, precipitated overnight at -20°C and resuspended in sterile water. The cDNA inserts were used as templates for large scale synthesis of a pool of RNAs to provide enough RNA for the subtractive hybridisation (a ten fold excess of MAA mRNA over the control testis cDNA is necessary). Linearised DNA (2-5μg) was incubated with 1 x RNA transcription buffer, 10mM DTT, 100U RNasin, 2.5mM each rATP, rCTP, rGTP and rUTP, and 20U T7 RNA polymerase in a reaction volume of 100μl. The reaction was carried out at 37°C for 1h, another 10U polymerase was added and the reaction continued for a further 1h. A 5μl sample of the reaction mix was separated on a 1% agarose gel in TBE to check synthesis of RNA. The remainder was precipitated with 0.1 volume 3M sodium acetate pH5.5 and 2.5 volumes cold absolute ethanol overnight at -20°C.

7.2.6 Subtractive hybridisation

Subtractive hybridisation was carried out mainly according to the method of Sive and St. John (1988).

Reverse transcription of control mRNA. Sertoli cell mRNA from control rat testis was purified as described above. This was reverse transcribed using a dT_{17} adaptor (I) primer (5'GGTCGACGGTACCGAATTCT(T_{17}) and the first strand cDNA synthesis reagents from the Stratagene Zap-cDNA synthesis kit following the manufacturers instructions as above. After completion of first strand cDNA synthesis the mRNA template was hydrolysed in 20mM EDTA pH8, 0.4% SDS and 0.36N NaOH with 2µg of muscle glycogen (Boehringer) at 68 °C for 30min. Samples were neutralised by addition of 1M Tris/Cl, pH7.6 and 1N HCl. The cDNA was size selected on a Sephacryl S-400 column and precipitated with 0.1 volumes 3M sodium acetate pH5.5 and 2.5 volumes absolute ethanol at -20°C overnight.

Photobiotinylation of MAA mRNA. RNAs transcribed from the MAA cDNA library was labelled with photoactivable biotin according to the manufacturer's instructions (Clontech, British Biotechnology, Cambridge). Briefly, photoactivable biotin (2µg) was added to 20µl aliquots of mRNA (0.5-0.7µg/µl) under subdued lighting in 1.5ml snap cap tubes. Tubes were placed on ice with lids open and the mixes irradiated for 10min with a UV sunlamp placed 10cm away. TE buffer was added to a volume of 100µl and the reactions extracted several times with an equal volume of water saturated 2-butanol until the discarded upper phase was colourless. The labelled mRNA present in the lower phase was precipitated with addition of 0.1 volumes 2M unbuffered sodium acetate and 2 volumes absolute ethanol at -20°C overnight.

Subtractive hybridisation. Single stranded control cDNA and a ten fold excess of the photobiotinylated MAA mRNA pool were co-precipitated with 3M sodium acetate and absolute ethanol at -70°C. The mRNA/cDNA mix was centrifuged at 12,000g for 15min and dissolved in 50µl hybridisation buffer (0.5M sodium phosphate pH7.2, 1M NaCl). This was overlaid with light paraffin oil, heated to 80°C for 5 min, cooled to 65°C and hybridisation allowed to proceed at this temperature for 48h. After this time additional biotinylated mRNA was added and hybridisation was allowed to continue for a further 24h.

After hybridisation was completed excess biotinylated mRNAs and mRNA: cDNA duplexes were removed by repeated extraction with phenol. Briefly, an equal volume of HB buffer containing 50mM HEPES and 2mM EDTA was added along with 200µg streptavidin. This was incubated at room temperature for 10min and extracted with an equal volume TE-buffered phenol:chloroform (1:1). The top aqueous phase was removed, HB buffer added to the organic phase and the extraction repeated. Aqueous phases were pooled and the extraction procedure was repeated twice more.

Finally, aqueous phases were precipitated on ice for 15min with 3M sodium acetate, pH5.5 and absolute ethanol. Subtracted cDNA was resuspended in 100μl STE and size selected on an S-300 spin column. cDNA was then precipitated and resuspended in 20μl sterile water.

Tailing and amplification of cDNAs. The resultant single stranded 'subtracted' cDNAs were tailed with dCTP (Davis *et al.*, 1986). The reaction mix contained 1 x terminal transferase buffer (10 x buffer contains 700mM Tris/HCl, pH7.6, 100mM $MgCl_2$ and 50mM DTT), 25mM dCTP and was catalysed by 15U terminal transferase at 37°C for 30min. Tailed cDNAs were dissolved in Taq polymerase buffer (50mM KCl, 10mM Tris/HCl pH9 at 25°C, 1.5mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X100) and made double stranded using a dG16 adaptor (II) primer (5' AGA ATT CGG TAC CGT CGA CC (G_{16}), 1μg) in the presence of 2.5U Taq polymerase (Perkin Elmer Cetus) and 100μM each dNTP (Pharmacia); incubation was 94°C for 2 min, annealing was 45°C for 2 min and extension was 72°C for 40 min .

The double stranded subtracted cDNAs were amplified by 'lone linker' PCR (Ko *et al.*, 1990). This was carried out using primer I without a dT tail (ie. 5' GGT CGA CGG TAC CGA TTC T) in a normal 100μl PCR reaction mix with 40 cycles of amplification; incubation was 94°C for 0.5min, annealing was 55°C for 1.0min and extension was 72°C for 3min. An aliquot of the reaction (12μl) was run on a 5% non-denaturing polyacrylamide gel to check amplification. PCR products were then purified, size selected using a S-400 cDNA column and stored at -20°C until labelling with ^{32}P-dCTP.

7.2.7 Screening of control Sertoli cell library

Plating of library and transfer to filters. XL1-blue cells for plating were grown in LB-broth, 10mM $MgSO_4$ and 0.2% maltose as described previously and were resuspended in 0.5 volumes cold, filtered 10mM $MgSO_4$. NZY agar plates (2 x 240mm^2) were poured and allowed to dry for at least 24h. XL1-blue cells (2 x 1ml) combined with approximately 2 x 10^5 plaques from the control Sertoli cell library (section 7.2.4) were incubated at 37°C for 20min, 40ml NZY top agarose at 48°C was added and this was poured onto NZY bottom agar plates prewarmed at 37°C. These were allowed to set for approximately 10min and then incubated inverted at 37°C for 16-20h until plaques developed.

When plaques had formed and were of the correct size (1-2mm), plates were placed at 4°C for at least 1h to allow the agar to harden. Duplicate lifts of the library were made onto nylon backed nitrocellulose filters (Hybond-C extra; Amersham)

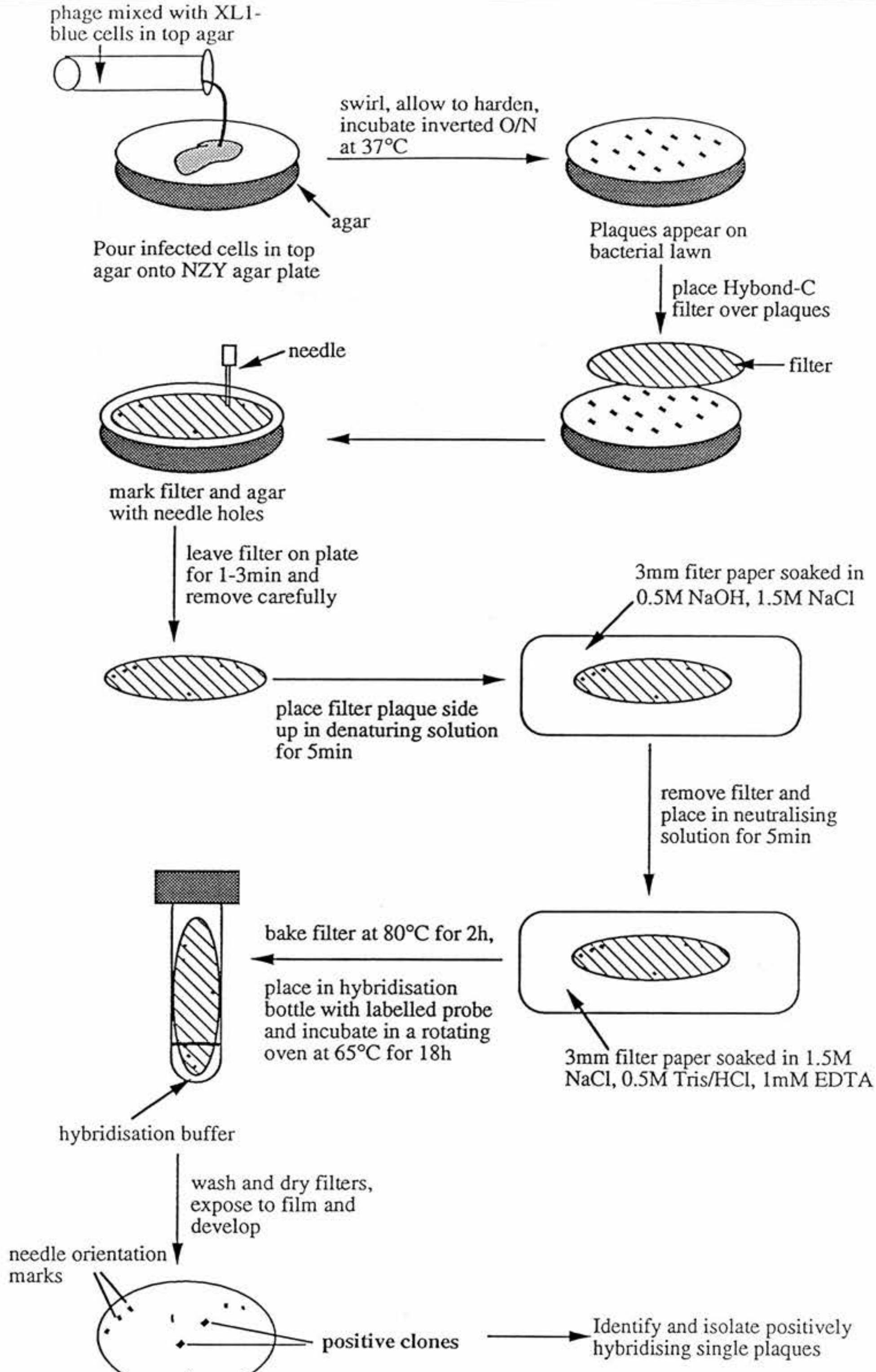

Figure 3. Outline of screening protocol. Diagram showing the methods used to plate and screen an enriched Sertoli cell cDNA library with a ^{32}P-labelled subtracted cDNA probe. *Adapted from Davis et al., 1986.*

according to the method of Davis *et al.*, 1986 (see Fig.3). Briefly, the first filter was laid onto the plate for 2min and orientation marks were made with a needle. The DNA on the membrane was denatured by placing it phage side up for 5min in 0.5M NaOH, 1.5M NaCl followed by neutralisation for 5min in 0.5M Tris pH7.5, 1.5M NaCl. A second lift was taken as above but the membrane was placed on the plate for 4min. The membranes were air dried for approximately 5min and the DNA was fixed onto the membrane by baking at 80°C for 2h.

Hybridisation of probe to filters. Subtracted cDNA (50ng) was labelled with ^{32}P-dCTP using the Amersham 'Multiprime' DNA labelling kit as described in chapter 3. Filters were prehybridised overnight at 65°C in buffer containing 0.2M sodium phosphate pH7.2, 1mM EDTA, 1% BSA, 7% SDS and 15% formamide. First and second lifts were placed in separate hybridisation bottles. Labelled cDNA was denatured, added to the bottles and hybridisation was allowed to continue for approximately 18h. Filters were washed for 2 x 30min at 65°C in buffer containing 40mM sodium phosphate pH7.2, 1mM EDTA and 1% SDS. Membranes were placed under X-Omat AR autoradiography film at -70°C for at least 24h. The films were developed and orientation marks on first and second lifts were lined up. Positively hybridising plaques present on both filters were identified and marked for analysis.

Isolation of positive clones. Single plaques or an area of agar containing several plaques in close association which were identified from the autoradiographs as hybridising to the subtracted cDNA probe were removed from the agar plates by coring out the top agarose using a 1ml pipette tip. This was placed into 1ml SM buffer in a glass tube and the phage were allowed to disperse by shaking at 4°C overnight.

NZY agar plates (90mm diameter) and fresh XL1-blue plating cells were prepared. Dilutions (1:10, 1:100, 1:1000) of the SM stocks of the primary positives were prepared using SM buffer, 5µl of each dilution was added to 200µl cells and plated as for the primary screening. Plates containing well spaced plaques were chosen to take duplicate lifts as before. The membranes were hybridised to labelled subtracted cDNA and positively hybridising plaques were identified. Individual plaques showing positive hybridisation were cored out of the agar and placed in 0.5ml SM buffer with 20µl chloroform. These were incubated overnight at 4°C before excision of plasmid DNA (see below).

A tertiary screen was carried out on positive signals from the secondary screen which corresponded to more than one plaque. The plaque containing area on the plate was cored out and placed in SM buffer. The screening was carried out as for the secondaries and single positive plaques were isolated.

In vivo excision of individual recombinant phagemids. LB-agar plates containing 100µg/ml ampicillin were poured. Fresh XL1-blue cells were grown in LB-broth containing 10mM $MgSO_4$ and were resuspended in 10mM $MgSO_4$ to an $O.D._{600}$ of 1.0. Cells (200µl) were combined with 200µl of selected SM phage stock (containing approximately 1 x 10^4 phage) and 2µl R408 helper phage (> 1 x 10^6 pfu/ml) and incubated at 37°C for 15min. Thereafter, 5mls of 2 x YT broth (10g NaCl, 10g yeast extract and 16g bacto-tryptone in 1 litre water) was added and incubated with shaking at 37°C for 3h. Cells were removed by heating at 70°C for 20min followed by centrifugation at 4000g for 5min and the supernatant containing phagemid as filamentous phage particles was stored at 4°C.

Supernatant (100µl) was combined with 100µl fresh XL1-blue cells, incubated at 37°C for 15min and 100µl was spread onto LB-amp plates which were incubated at 37°C overnight. Individual bacterial colonies were picked from these plates and restreaked onto numbered segments of LB-amp plates. These were again incubated at 37°C overnight.

7.2.8 Analysis of positive clones

The strategy used for analysis of clones hybridising to subtracted cDNAs is outlined in Fig.4.

Direct lysis PCR of plasmids. Single colonies from the restreaked rescued phagemid were picked (approximately 3 from each original rescue), placed into 50µl lysis buffer (contains 20mM Tris/HCl, pH8.5, 2mM EDTA, 1% Triton-X-100), heated at 98°C for 5min and the cell debris removed by centrifugation at 12,000g for 5min. PCR was carried out under normal reaction conditions (see chapter 3, section 6) using 5µl of the supernatant as a template. Primers used for amplification were directed against T3 and T7 RNA polymerase sites present on the Bluescript vector (as described in chapter 3) and 40 cycles of amplification were carried out; melting was for 0.5min at 94°C, annealing was 1min at 45°C and extension was 3min at 72°C. A 10µl aliquot of each PCR reaction was separated on a 1% agarose gel run in parallel with DNA markers and the size of the cDNA inserts were determined. Amplified cDNA was isolated from the PCR reaction mixes using Clontech Chroma Spin +TE-100 columns.

Southern blotting. DNAs amplified by direct lysis PCR were separated by electrophoresis and then transferred to nylon membranes (Hybond-N; Amersham) by Southern blotting. Briefly, agarose gels were incubated in denaturing solution (0.5M NaOH, 1.5M NaCl) for 20min at room temperature on a rocker platform, placed on a plastic tray and the membrane was laid directly onto the gel and covered with several layers of 3mm filter paper and paper towels. A heavy weight was placed on top and

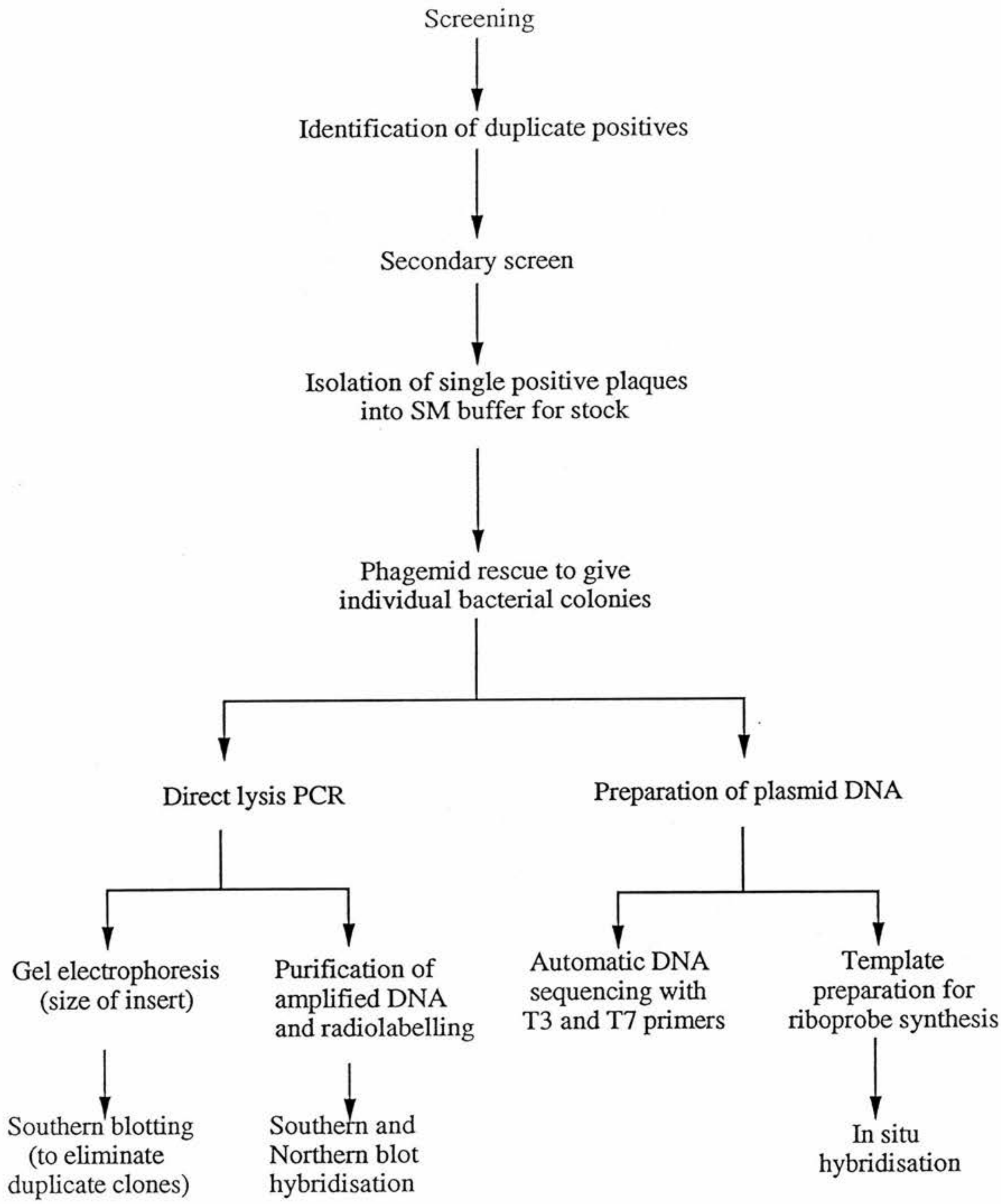

Figure 4. Analysis of positive clones. Flow chart outlining the techniques used to analyse clones isolated by screening of the control cDNA library with ^{32}P-labelled subtracted cDNAs.

transfer by capillary action was allowed to continue for 3-6h at room temperature. DNA was fixed to the membrane by UV crosslinking and the blot was stored in clingfilm until hybridisation. Membranes were hybridised to ^{32}P-dCTP labelled positive cDNA inserts to identify duplicate clones using standard conditions.

Northern blot analysis. Control, MAA + 21d and MAA + 42d testis RNA (15μg each) were separated on a denaturing agarose gel as described in chapter 3, section 3. RNA was transferred to a membrane (see chapter 3, section 7), fixed and hybridised to ^{32}P-dCTP labelled positive cDNA inserts (see chapter 3, section 6). This enabled size determination of the corresponding mRNA and initial evaluation of its modulation by spermatids.

Plasmid preparation and digestion. Single colonies from the restreaked positive clones were used to inoculate LB-broth containing 50μg/ml ampicillin and were cultured overnight at 37°C. Glycerol stocks were taken and plasmid DNA was prepared using the Promega 'Magic Minipreps' method. Plasmid DNA was digested with Kpn I or Bam HI to give linearised templates for preparation of antisense or sense riboprobes, respectively. All methods were as described in chapter 3, sections 5 and 8.

In situ hybridisation. ^{35}S-UTP labelled riboprobes were prepared from the linearised templates as described previously (chapter 3, section 8). Radioactive *in situ* hybridisation was carried out on testicular sections from control, MAA+21d and MAA+42d adult rats as described in chapter 3, section 9.

Sequencing of positive cDNA's. Plasmid DNA prepared by the Magic Minipreps method was sequenced in both directions using T7, T3 and SK primers in an automatic sequencing reaction (chapter 3, section 12). Sequence data was analysed and error rate determined using the Genejockey computer program and vector sequences removed prior to determination of homology to any known sequence lodged in Genbank using the wordsearch package on the Daresbury Vak (Wisconsin GCG package at the Daresbury Seqnet Service).

7.3. Results

7.3.1 Cell fraction analysis

Immunostaining of a sample of isolated cell fraction 4 for the presence of SGP-1 confirmed the presence of Sertoli cells in this fraction (see Fig.5). Contaminating cells were mainly elongate spermatids.

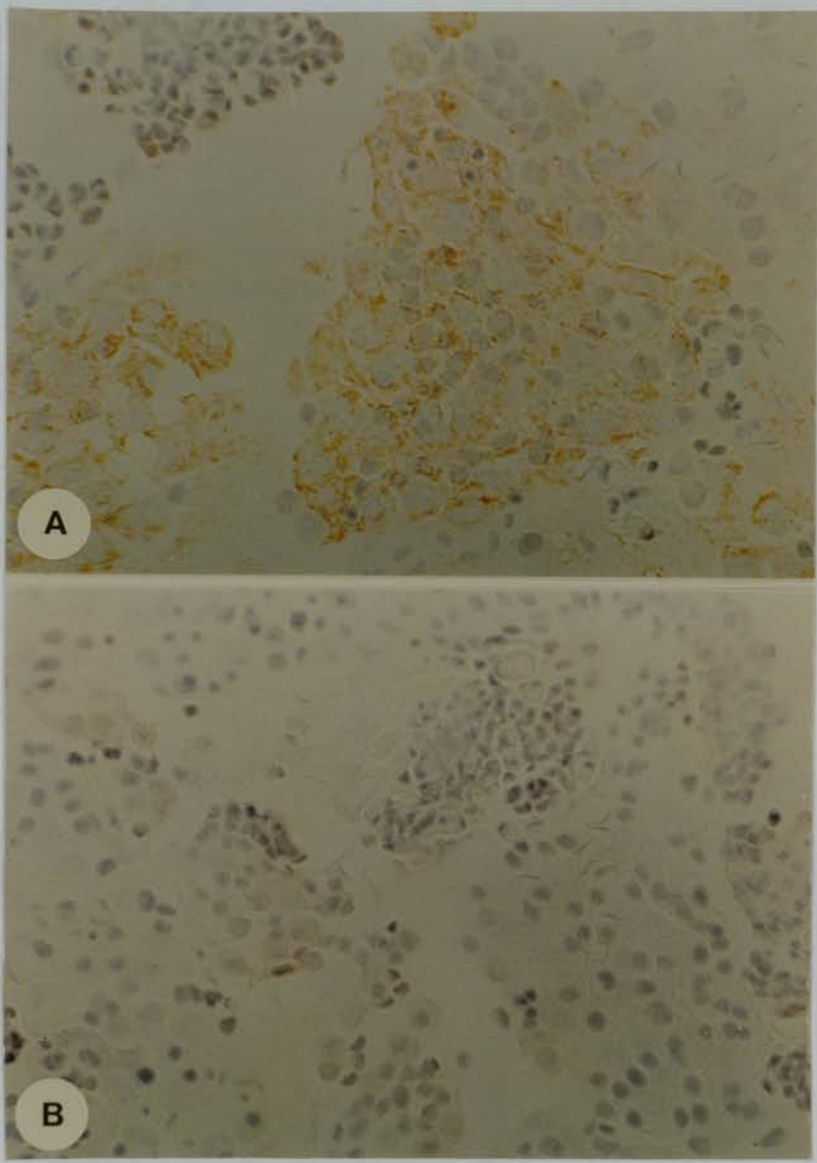

Figure 5. SGP-1 localisation in cell fraction. Immunostaining of fraction 4 with (A) anti-SGP-1 antibody and (B) normal rabbit serum (negative control; diluted1:1000 in TBS). Cells in (A) stained orange for SGP-1 are Sertoli cells. Magnification x363.

7.3.2 cDNA synthesis

Synthesised cDNA was visualised by electrophoresis through a 5% polyacrylamide gel (Fig.6). The cDNA was fractionated into three samples by size selection using a Sepharose S400 column. Complementary DNA in fraction 1 which had been synthesised from control rat testis mRNA (Fig.6, lane C1) was observed to contain a variety of long length cDNAs, of approximately 700 - 3000bps. This would suggest that there are full length cDNAs in this group. The second fraction contained a smaller amount of cDNA of these lengths and some shorter cDNAs of approximately 200-300bps (Fig.6, lane C2). Finally, the third fraction collected did not contain significant amounts of large cDNAs (Fig.6, lane C3). This pattern was also found in the fractions of cDNA synthesised from mRNA which had been purified from Sertoli cells isolated from rats 21 days after MAA treatment (Fig.6, lanes M1-M3).

7.3.3 Titre and quality of libraries

The titres of the libraries were determined as outlined in the methods section and the data is presented in Table 1.

Table 1. Titres of cDNA libraries.

Library	Titre (pfu/ml)
Control	7.3×10^5
MAA + 21d	4.4×10^5
Control - amplified	5.0×10^9
MAA + 21d - amplified	4.1×10^9

Titres of primary and amplified libraries prepared as described in the methods section. Data is shown for control Sertoli cell enriched cDNA library and the cDNA libraries prepared from Sertoli cells isolated from rats 21d after MAA treatment.

The sizes of selected cDNAs present in the amplified libraries were determined to establish that the recombinant lambda contained a variety of cDNAs of varying sizes. This was achieved by direct lysis PCR of cDNA inserts in Bluescript rescued from the libraries. Gel analysis revealed that in the control amplified library there were inserts of a variety of sizes ranging from 500-2000bps (see Fig.7a). The MAA + 21d amplified library showed a similar pattern, with inserts of approximately 400-2000bps (see Fig.7b).

7.3.4 Identification of positive clones

Out of approximately 4×10^5 plaques screened with the subtracted cDNA probe a total of 17 single plaques were identified after either two or three rounds of screening

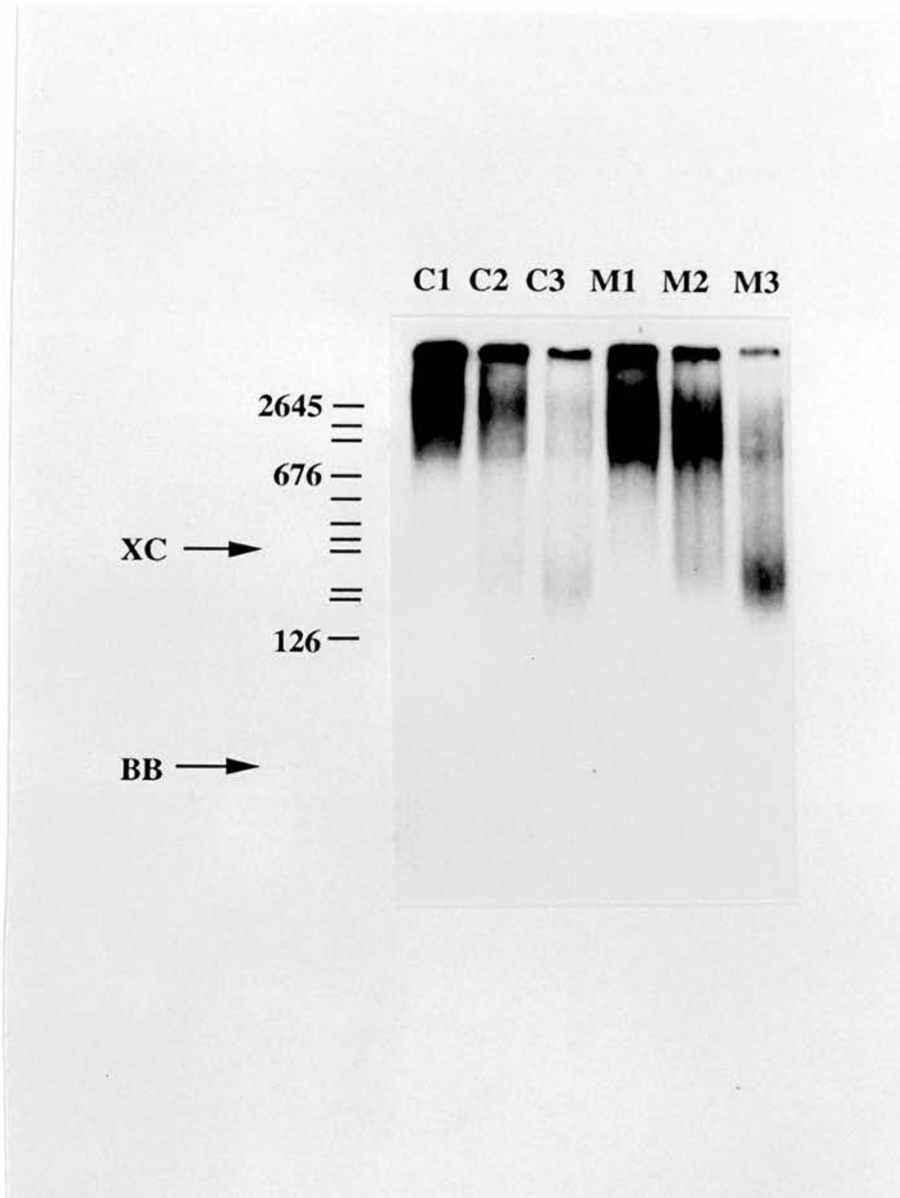

Figure 6. Analysis of synthesised cDNA. Polyacrylamide gel analysis of cDNA synthesised from control and MAA + 21d Sertoli cell enriched mRNA. Three fractions collected after size selection with a S-400 cDNA column are shown. Markers are pGem (Promega) with sizes of 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 and 126bps. C = control cDNA; M= MAA + 21d cDNA; XC = xylene cyanol dye front (260bps); BB = bromophenol blue dye front (65bps).

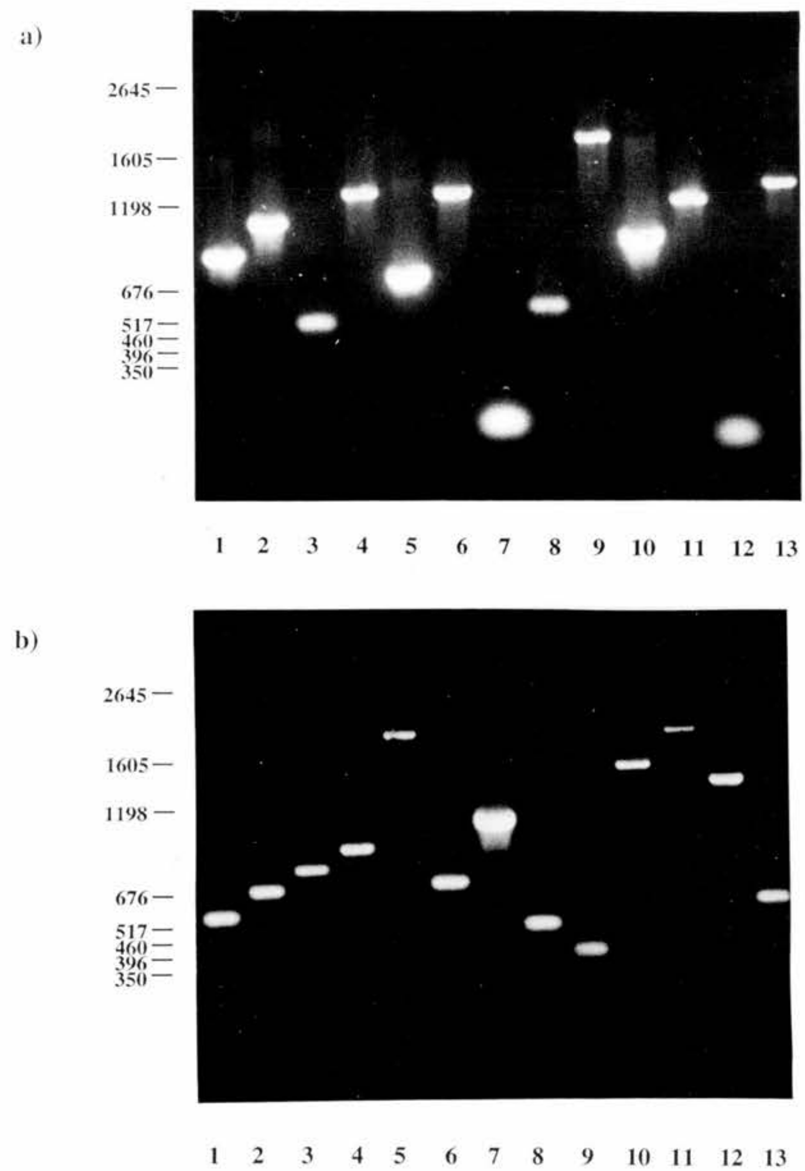

Figure 7. Illustration of size of cDNA inserts in library. Direct lysis PCR of randomly selected Bluescript clones rescued from a control (A) and MAA + 21d (B) Sertoli cell enriched cDNA libary using T3/T7 primers. PCR products were separated on 1% agarose gels with pGem markers (Promega) of the size in bps shown.

as giving a positive signal on hybridisation. These plaques were isolated and plasmid DNA was purified following the Bluescript rescue procedure. Direct lysis PCR was carried out on the individual clones to determine the size of the cDNAs; sizes ranged from 700-2600bps. Many of the inserts were found to be identical in size and Southern blotting was carried out to eliminate duplicate clones from the analysis. Two clones were selected for use as probes on Southern blots; these were named M2.10B5 and M2.21B5 and had a length of 1200bp and 2600bp, respectively. A representation of the typical result obtained from these Southern blots is shown in Fig.8. Several clones appeared to contain inserts with significant sequence homology and therefore only 4 unique clones were ultimately chosen for further analysis.

7.3.5 Analysis of selected positive clones

Northern blot and in situ hybridisation analysis was performed to obtain more information about the selected clones. DNA sequencing of the clones was also performed to determine any sequence homologies. Table 2 outlines the results of these analyses.

Table 2. Characterisation of positive clones.

Clone	Northern blot analysis	In situ hybridisation	Sequence data
M2.10B5	strong signal approx. 1kb	very abundant, stage specific, localised in germ cells and possibly Sertoli cells	identity with part of the mitochondrial genome encoding NADH dehydrogenase subunit II and tRNA-Met
M2.21B5	weak signal approx. 2.5kb	N.D.	no good sequence data obtained
M2.22B1	N.D.	N.D.	no significant homologies
M3.20A8	very weak signal approx. 2.5kb	N.D.	no consistent sequence homologies apart from foetal expressed sequence tags

Outline of the analysis of selected clones identified by subtractive hybridisation. Transcript sizes and abundance shown by Northern blot analysis. Cellular localisation and stage specificity demonstrated by in situ hybridisation. Sequence homologies are also shown. N.D.= none detectable.

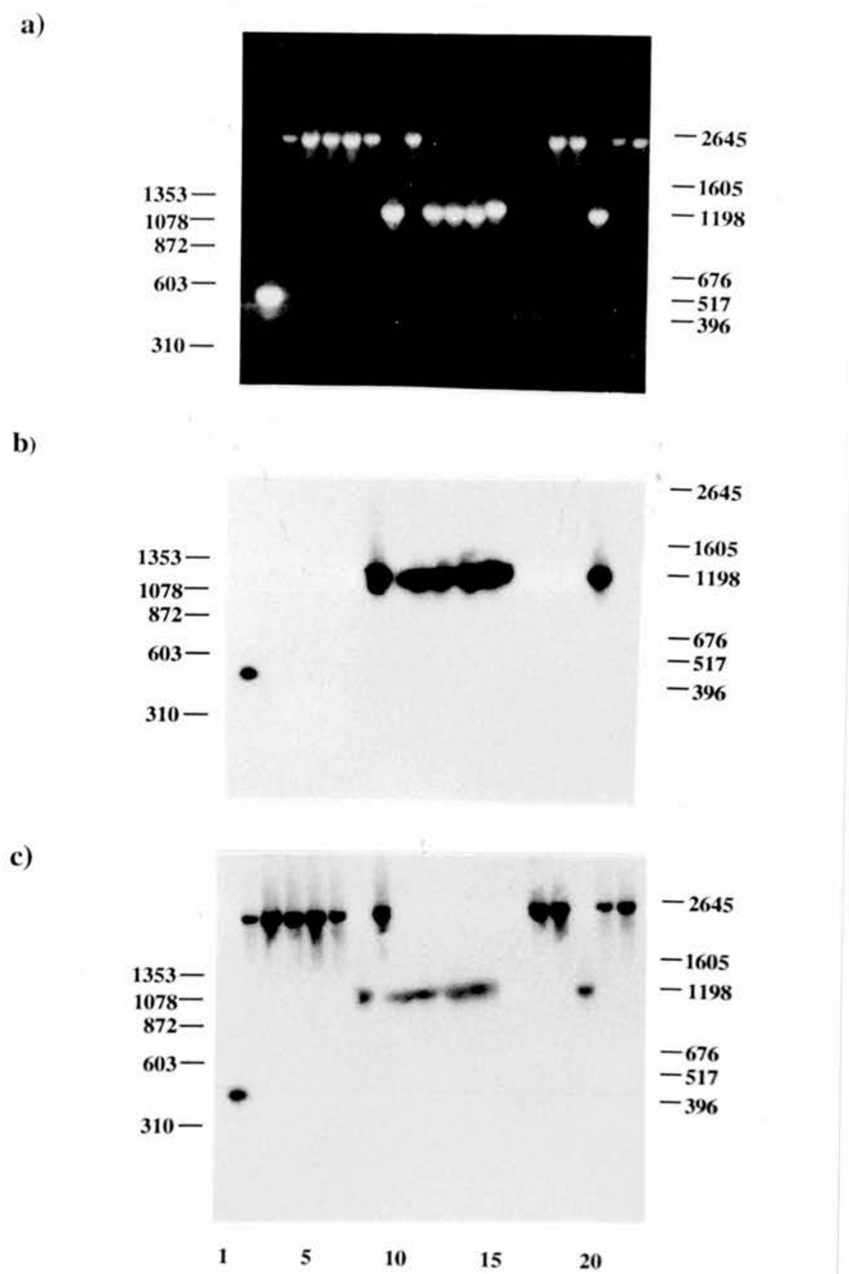

Figure 8. Identification of duplicate clones. (A) Direct lysis PCR of selected Bluescript clones rescued from plaques positively hybridising to the subtracted cDNA probe. Markers shown are Hae III phi X (left hand side; IBI) and pGem (right hand side; Promega). (B) Autoradiograph of a Southern blot of the agarose gel in (A) probed with ^{32}P-labelled positive clone M2.10B5 and showing several clones with identity to M2.10B5. (C) Southern blot in (B) stripped and reprobed with ^{32}P-labelled positive clone M2.21B5 and showing several clones with identity to M2.21B5. Autoradiograph in (C) shows residual radioactivity remaining from (B) due to incomplete stripping of the membrane.

Northern blot of total testis RNA probed with M2.10B5 showed an abundant, low molecular weight transcript of approximately 1kb (Fig.9a). The level of expression of this transcript did not appear to be affected by the depletion of elongate spermatids at 21 days after MAA treatment. Hybridisation with radiolabelled M2.21B5 gave a weak signal on Northern blot showing a transcript of approximately 2.5kb (Fig.9b). Again, there was no obvious change in the signal obtained from RNA isolated from testis at MAA + 21d. Northern blot with M2.22B1 had not detected a transcript in the testis after 2 weeks exposure of the autoradiograph. When M3.20A8 was used as a probe a very weak signal for a transcript greater than 2kb was detected with no visible change in the level of expression at 21 days after MAA treatment (data not shown).

In situ hybridisation was carried out using the four selected clones as riboprobes but a signal was detected only with M2.10B5. The transcript appeared to be abundantly expressed and was localised mainly to the germ cells of the seminiferous epithelium, in particular the pachytene and diplotene spermatocytes (Fig.10). The distribution of the mRNA appeared to be stage specific probably due to the changing complement of these germ cells at each stage of the spermatogenic cycle, with a more diffuse expression at the later stages of the cycle which contain diplotene spermatocytes. It was not possible from the radioactive *in situ* hybridisation undertaken to determine if the transcript was also expressed in Sertoli cells.

Sequencing of clone M2.10B5 generated over 400 bases of sequence with a low error rate (see Fig.11). Comparison of this data with sequences lodged in Genbank showed very good homology with part of the rat (Rattus norvegicus) mitochondrial genome coding for transfer RNA-Methionine and NADH dehydrogenase subunit II (Fig.12). Sequence data was obtained for clone M3.20A8 (Fig.13) but did not reveal any consistent homologies with known sequences with the exception of short sequences of foetal brain expressed sequence tags (sequence data not shown). Sequencing of clone M2.22B1 showed no homology with any sequence in Genbank. Finally, no good sequence data was obtained for clone M2.21B5.

7.4. Discussion

Subtractive hybridisation is a very useful technique for the identification and cloning of genes which are differentially regulated. The procedure has been used successfully to isolate T cell-specific membrane-associated proteins (Hedrick *et al.*, 1984), to identify RNAs specifically expressed in scrapie-infected brains (Duguid *et al.*, 1988), to clone genes under the control of the circadian clock in *Neurospora* (Loros *et al.*, 1989), and in many other systems. The technique of subtractive

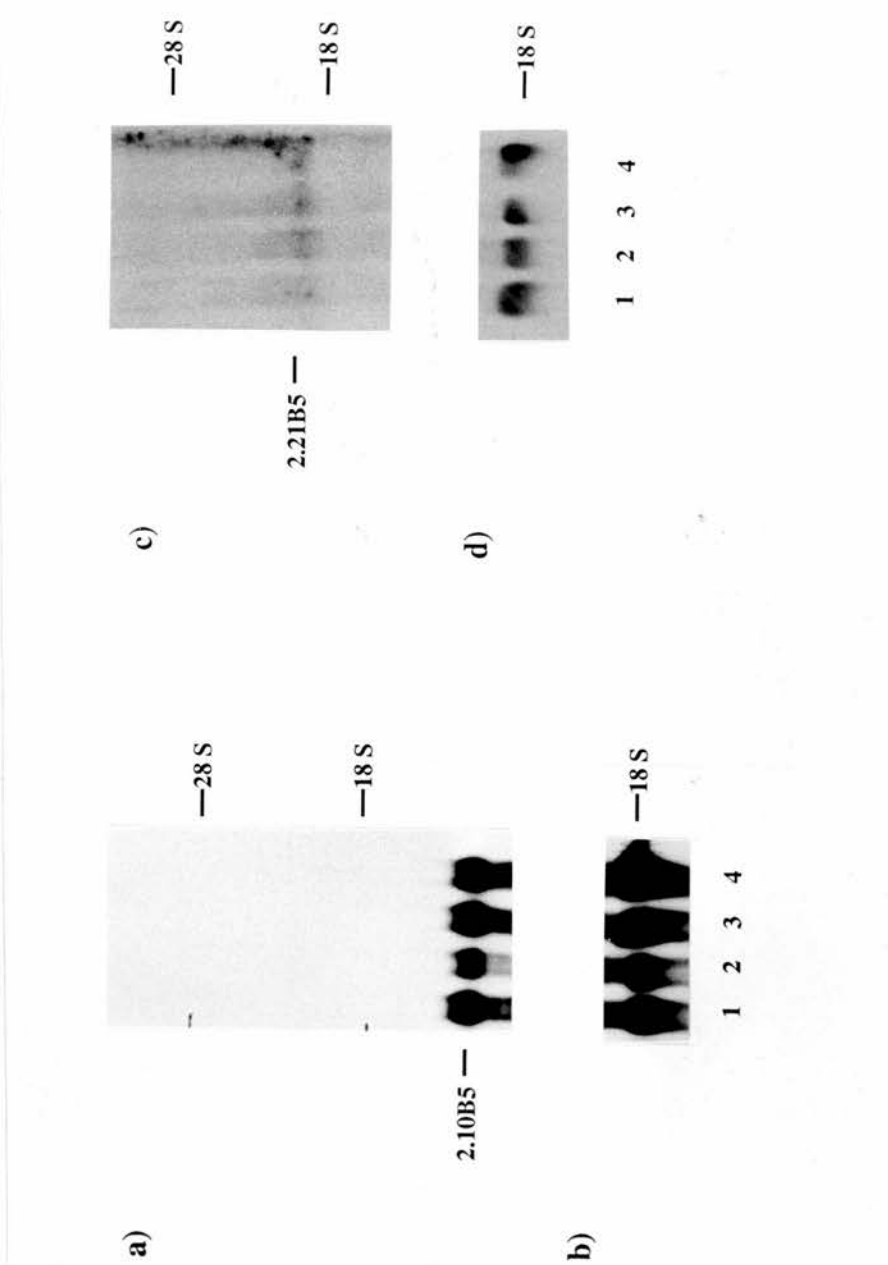

Figure 9. Northern blots of selected positive clones. Samples are from a control rat testis (lanes 1 and 4), and testes from animals administered a single oral dose of methoxyacetic acid 21 or 42 days previously (lanes 2 & 3, respectively). All lanes were loaded with 15μg total RNA. The membrane was hybridised with ^{32}P-labelled M2.10B5 (A) or M2.21B5 (C) cDNAs. The membranes were stripped and reprobed with an oligonucleotide probe for the 18S ribosomal RNA to check that all lanes contained equivalent amounts of RNA (B & D).

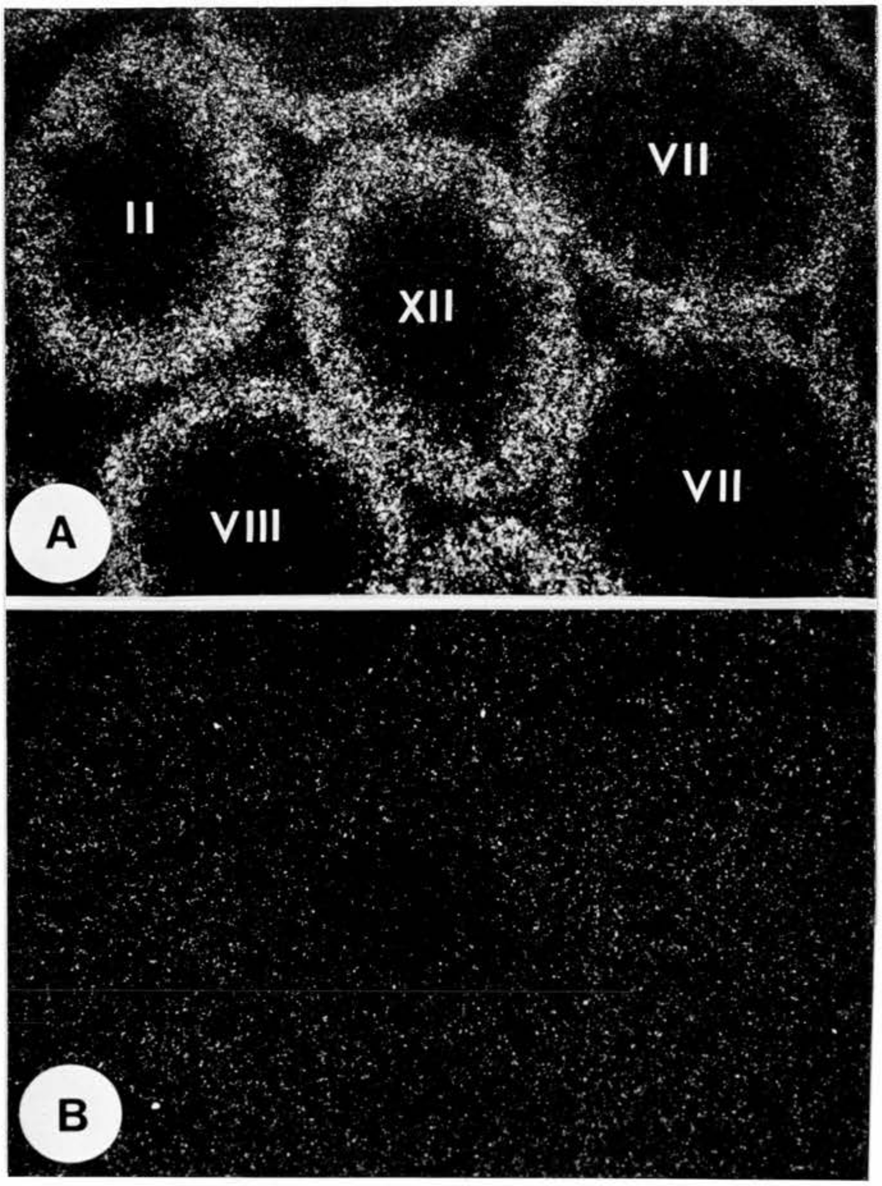

Figure 10. Cellular localisation of M2.10B5. *In situ* hybridisation of antisense radiolabelled M2.10B5 riboprobe on control rat testis showing possible localisation of transcript in germ cells and stage specificity of expression (A). Control rat testis section hybridised to sense M2.10B5 riboprobe showing no specific hybridisation (B). x90 magnification.

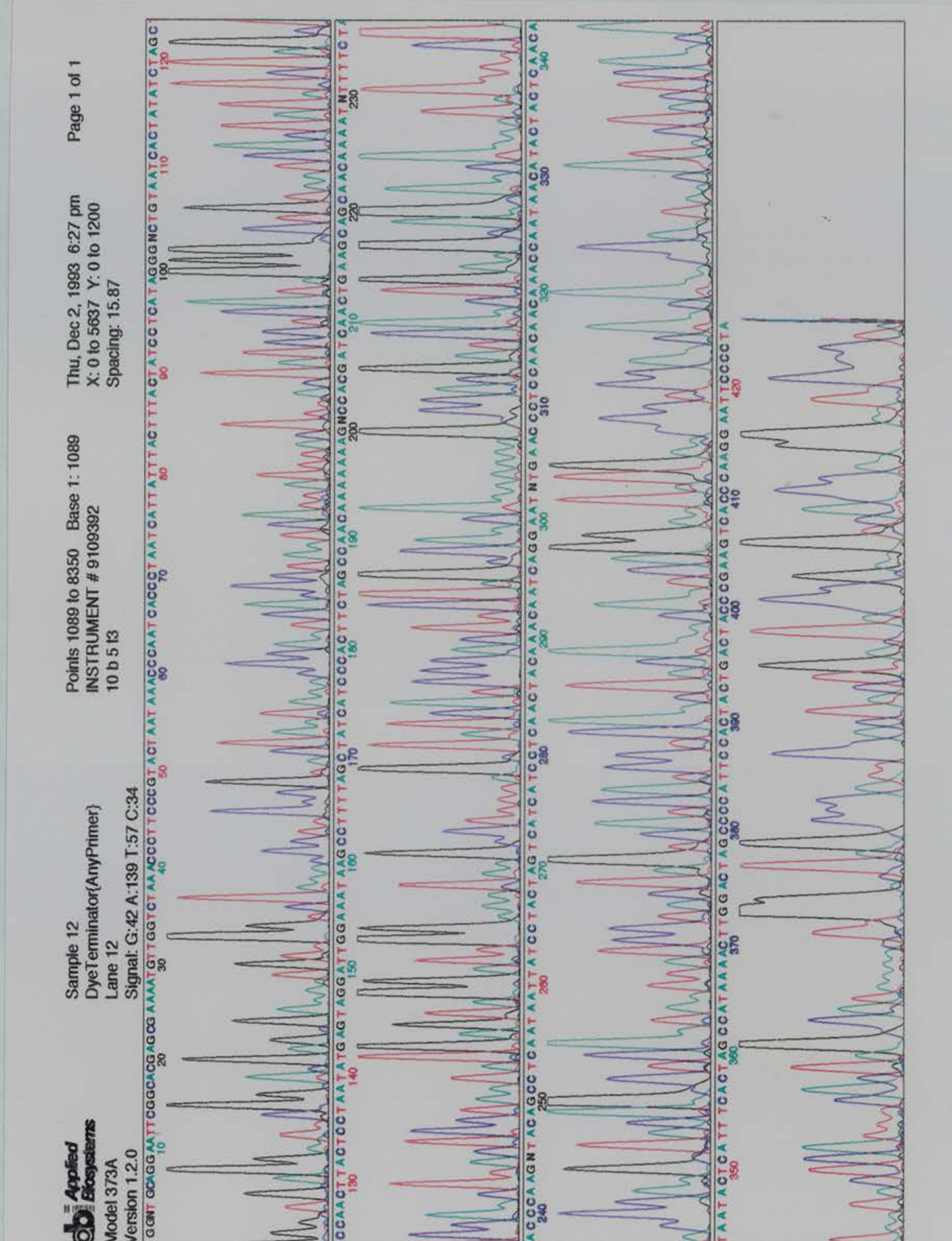

Figure 11. Sequence data for clone M2.10B5. Print out of scan obtained from automatic sequencing of M2.10B5 showing data obtained.

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              .         .         .         .         .
  12 CGAAAATGTTGGTCTAAACCCTTCCCGTACTAATAAACCCAATCACCCTA 61
     ||||||||||||| ||||||||||||||||||||||| ||||| ||||||
3861 CGAAAATGTTGGT.TAAACCCTTCCCGTACTAATAAATCCAATTACCCTA 3909

              .         .         .         .         .
  62 ATCATTATTTACTTTACTATCCTCATAGGGNCTGTAATCACTATATCTAG 111
     | |||||||||||| || | | | | |||     ||||||| | |  |||
3910 ACCATTATTTACTTAACCACCTTTAAAGGCCGCCTAATCACGACACTTAG 3959

              .         .         .         .         .
 112 CTCCAACTTACTCCTAATATGAGTAGGATTGGAAATAAGCCTTTTAGCTA 161
     | |||||||||  | |||||||||||||||||||||||||||||||||||
3960 CACCAACTTACCACCAATATGAGTAGGATTGGAAATAAGCCTTTTAGCTA 4009

              .         .         .         .         .
 162 TCATCCCACTTCTAGCCAACAAAAAAAGNCCACGATCAACTGAAGCAGCA 211
     |||||||||||||||||||||||||||| |||||||||||||||||||||
4010 TCATCCCACTTCTAGCCAACAAAAAAAGCCCACGATCAACTGAAGCAGCA 4059

              .         .         .         .         .
 212 ACAAAATNTTTTCTAACCCAAGNTACAGCCTCAATAATTATCCTACTAGT 261
     ||||||| |||||||||||||| |||||||||||||||||||||||||||
4060 ACAAAATATTTTCTAACCCAAGCTACAGCCTCAATAATTATCCTACTAGT 4109

              .         .         .         .         .
 262 CATCATCCTCAACTACAAACAATCAGGAATNTGAACCCTCCAACAACAAA 311
     |||||||||||||||||||||||||||||| |||||||| ||||||||||
4110 CATCATCCTCAACTACAAACAATCAGGAATATGAACCCTTCAACAACAAA 4159

              .         .         .         .         .
 312 CCAATAACATACTACTCAACATAATACTCATTTCACTAGCCATAAAACTT 361
     ||||||||||||||||||||||||||||||||||||| ||||||||||||
4160 CCAATAACATACTACTCAACATAATACTCATTTCACTGGCCATAAAACTT 4209

              .         .         .         .         .
 362 GGACTAGCCCCATTCCACTACTGACTACCCGAAGTCACCCAAGGAATTCC 411
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4210 GGACTAGCCCCATTCCACTACTGACTACCCGAAGTCACCCAAGGAATTCC 4259
```

Figure 12. Sequence alignment of M2.10B5 and part of rat mitochondrial genome. Top line is clone M2.10B5 sequenced with SK primer; bottom line is bases 3861-4259 of the rat (Rattus norvegicus) mitochondrial genome. Bases 3824-3891 code for tRNA-Met and bases 3892-4929 code for NADH dehydrogenase subunit II.

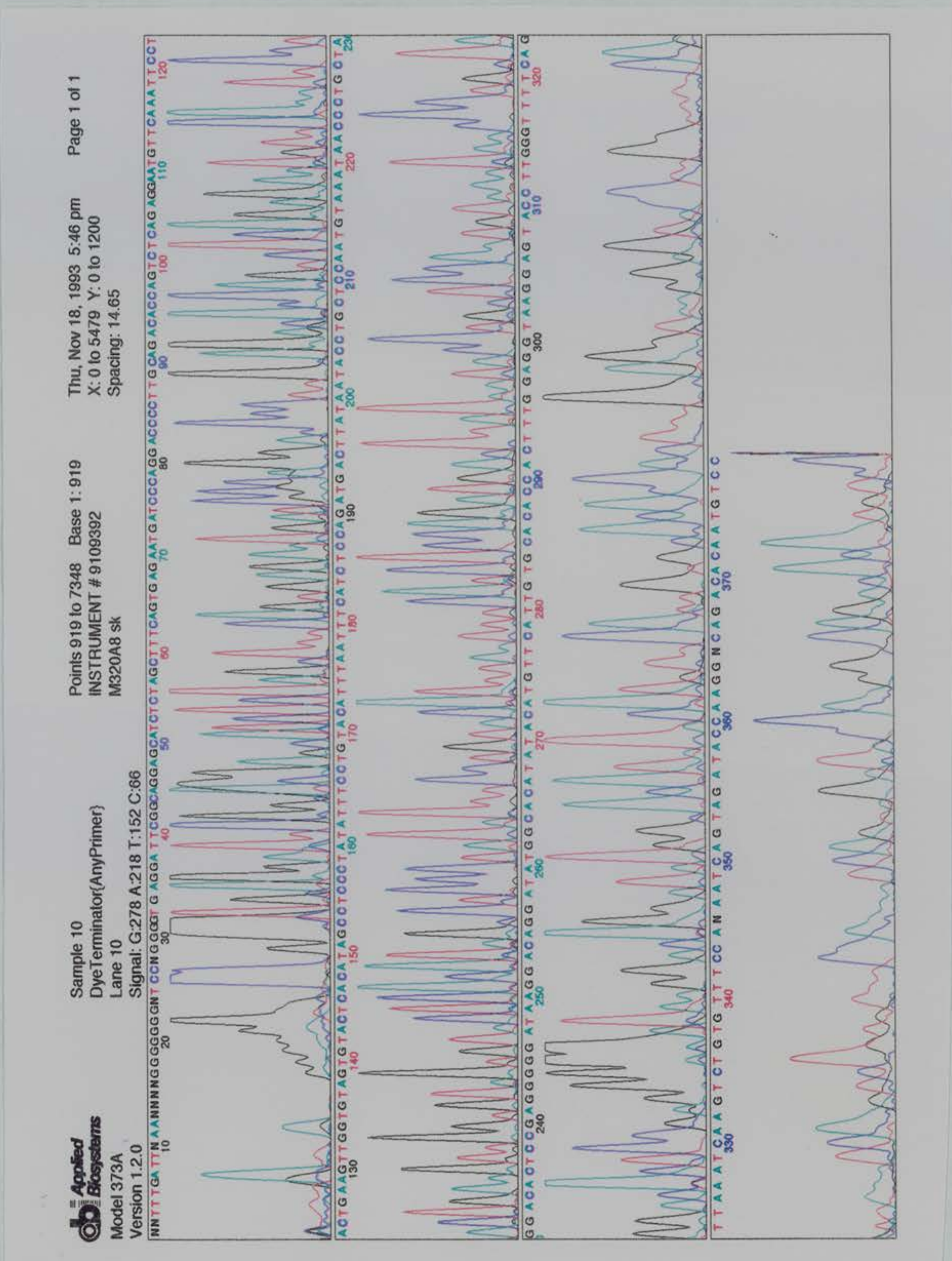

Figure 13. Sequence data for clone M3.20A8. Print out of scan obtained from automatic sequencing of M3.20A8 showing data obtained.

hybridisation has been modified quite extensively in recent years to allow easier separation of common hybrid sequences from the unique subtracted cDNAs (Sive & St. John, 1990). This modification involved separation of biotinylated mRNA from untreated cDNA by the use of streptavidin and phenol extraction. The technique is much simpler than those previously used, such as hydroxyapatite chromatography, and results in an increased yield of subtracted material. The population of cDNAs which it was the aim of this study to identify, ie. those expressed in Sertoli cells which are positively regulated by elongate spermatids, may be very small and therefore it was necessary to increase the yield of these cDNAs to allow the use of the DNA for screening of cDNA libraries. The application of lone-linker PCR for amplification of the subtracted cDNA has fulfilled this requirement (Saiki *et al.*, 1988; Ko *et al.*, 1990). The subtraction procedure requires that ten times as much mRNA from one tissue is used to "subtract" from cDNA prepared from the other sample. In the past this requirement often meant that the technique was not practical for tissues which were only available in small quantities. The present study used single stranded RNAs prepared from an enriched fraction of Sertoli cells from adult rat testis of animals previously treated with MAA. The amount of mRNA obtained from these cells is not substantial and therefore it would normally be necessary to treat and sacrifice many animals to obtain the required quantity of mRNA. However, this was overcome by the preparation of a unidirectional cDNA library from the original mRNA. From this library it was possible to carry out a large scale synthesis of single stranded RNAs to obtain the material required.

The aim of this study was to isolate specifically Sertoli cell mRNAs whose expression is modulated by elongate spermatids. From previous studies it has been established that many germ cell mRNAs including those encoding the protamines and transition proteins are very abundantly expressed (Hecht, 1990). It is known that heavily expressed messages will often not be "subtracted" out during the procedure and many of the clones isolated will simply be abundant mRNAs which are not differentially expressed (Saunders *et al.*, 1992; 1993). To avoid this complication mRNA was not isolated from total testis tissue but from a cell fraction which was selected to be enriched in Sertoli cells (Meistrich *et al.*, 1981). This fraction was used as a source of mRNAs for synthesis of cDNA used during subtractive hybridisation and the subtracted cDNAs were then used to screen a cDNA library prepared from a Sertoli cell enriched cell fraction from control rats to enhance the chances of isolating mRNA expressed in Sertoli rather than germ cells.

However, it is extremely difficult to isolate a pure population of Sertoli cells from the adult rat testis. Traditionally most groups have used immature rats from which to obtain a pure population of Sertoli cells. In 20 day old rats the most advanced population of germ cells present in the seminiferous tubules are early pachytene spermatocytes (Russell *et al.*, 1987). These cells are easily removed from their associations with the Sertoli cells (Romrell & Ross, 1979) resulting in an almost pure population of Sertoli cells for study. However, the response of Sertoli cells to hormones such as FSH and testosterone changes with the age of the animal from which the cells were isolated (Steinberger *et al.*, 1978) and it has also been demonstrated that the pattern of proteins secreted from immature seminiferous tubules in culture differs from that of the adult seminiferous tubule (McLaren *et al.*, 1993). Taken together these results demonstrate that the use of immature Sertoli cells might not always provide an adequate model for the study of spermatogenesis in the adult testis. Therefore a modification of the method of Meistrich and co-workers (1981) was used to isolate a population of adult rat cells enriched in Sertoli cells. This cell fraction was contaminated mainly with elongate spermatids which are extremely difficult to remove from Sertoli cells because of the presence of several specialised junctions between the two cell types (see Russell, 1993a). A recent study reported an improved method for the isolation of Sertoli cells from the adult rat testis and claimed to achieve a >90% pure population (Karzai & Wright, 1992). However, this technique is based on gravity sedimentation and therefore is extremely time consuming. The present study requires that the cells be isolated as quickly as possible to prevent any major changes in the RNA population present at the time of the animals death and to allow purification of intact RNA.

The pattern of expression of mRNA hybridising to the clone M2.10B5 *in situ* was not consistent with the transcript being present only in Sertoli cells. Localisation of the silver grains appeared to be mainly in germ cells with the most intense signal over the pachytene spermatocytes. Sequence analysis of this clone showed that it was almost identical to part of the rat mitochondria genome coding for NADH dehydrogenase subunit II and the transfer RNA for methionine (tRNA-Met). NADH dehydrogenase is part of the electron transport chain in mitochondria. It is involved in the transfer of electrons derived from intermediates of the tricarboxylic cycle and other substrates to oxygen, the final electron acceptor in respiration. The 29 nucleotide differences between clone M2.10B5 and tRNA-Met/NADH dehydrogenase subunit II in over 400 bases of sequence data may be due to species variation. The mitochondrial genome is circular and transcription of the genes contained within it is initiated only at two points

(Clayton, 1991). The RNA produced lacks introns and is processed after transcription to separate the individual mRNAs and tRNAs. This method of transcription explains why the clone M2.10B5 had homology to two genes as NADH subunit II and tRNA-Met are adjacent to each other on the mitochondrial genome (Darnell *et al.*, 1986). Sequencing of the clone gave approximately 30 bases with identity to tRNA-Met while the remainder of the clone had homology to the NADH dehydrogenase subunit II. This may explain why only one species of mRNA was detected on Northern blot of total testis RNA. This was approximately 1kb and corresponds to the size of the NADH dehydrogenase subunit II mRNA (Binder *et al.*, 1992). The high level of expression of NADH dehydrogenase subunit II in pachytene spermatocytes may be due to an increase in energy demands as they enter the final stages of meiosis. The high level of expression of mRNA coding for cytochrome c oxidase II, another member of the electron transport chain, in pachytene spermatocytes may be consistent with this interpretation (Saunders *et al.*, 1993). It is known that subtractive hybridisation techniques commonly isolate genes coded for by the mitochondrial genome (Shan *et al.*, 1990; Ku *et al.*, 1991; Saunders *et al.*, 1993). The reason for this is unknown but may be due to the fact that mitochondrial DNA is present at a very high cellular copy number of 10^3-10^4 copies per somatic cell (Clayton, 1991).

Clone M3.20A8 hybridised to a mRNA transcript in the testis which appeared to be expressed only at very low levels. This transcript could not be identified by *in situ* hybridisation. Analysis of data obtained from sequencing of this clone showed no consistent homologies apart from with foetal brain expressed sequence tags (ESTs). These are simply segments of sequence from cDNA clones which correspond to mRNAs (Adams *et al.*, 1991). The clones were selected randomly from human brain cDNA libraries and were partially sequenced. Some sequence homologies for these ESTs were found in Genbank but they are useful mainly as markers on the human genome for identification of genes involved in genetic diseases. This technique identified a large number of genes coded for on the mitochondrial genome again highlighting the high probability of isolating mitochondrial sequences from a cDNA library.

The experimental procedure used in these studies could be altered to enhance the probability of isolating spermatid regulated Sertoli cell genes. An improved method of Sertoli cell isolation from adult rats is necessary to eliminate identification of germ cell mRNAs. Sertoli cells are probably best isolated from tubules in which the germ cells are not needed as isolation procedures for Sertoli cells and germ cells require different approaches. To isolate germ cells it is necessary to subject the testicular tissue to harsh enzymatic treatments to break the junctional specialisations between the Sertoli cells and germ cells. However, this treatment does not allow isolation of intact Sertoli cells which can only be achieved if these cells are separated from the other cells of the testis while in clumps, without rough enzymatic treatment.

The studies described in this chapter were undertaken with the aim of isolating genes coding for either known or novel proteins in Sertoli cells, the expression of which is regulated by elongate spermatids. The initial studies described failed to identify any such genes but further analysis of the clones isolated may provide interesting information about gene expression in the testis. Also, another round of subtractive hybridisation and screening of the cDNA library may yield the desired results. However, it is possible that elongate spermatids do not have a large role to play in the control of Sertoli cell gene expression. To my knowledge, the studies reported in chapter 6 demonstrating the positive regulation of CP-2 mRNA expression in Sertoli cells by elongate spermatids are the first such reports of elongate spermatid influence on gene expression at the level of transcription. Therefore, the population of cDNAs which I aimed to identify may be extremely small making it difficult to isolate any without improved subtraction and cloning methods. In contrast, it has been shown in several studies that elongate spermatids do have an influence on Sertoli cell secretion of proteins such as inhibin (Allenby *et al.*, 1991) and ABP (Pineau *et al.*, 1989). This may suggest that elongate spermatids exert their effect on Sertoli cell function at a post-transcriptional level either influencing stability or translation of the mRNA, post -translational modifications or the rate of secretion of the proteins. However, the work undertaken in this study requires much more investigation before an important role of elongate spermatids on Sertoli cell gene expression can be excluded from the complex cellular interactions occurring in the testis.

8. Discussion

The aim of the work described in this thesis was to acquire a further understanding of the interactions between Sertoli cells and germ cells which are essential for maintaining the normal process of spermatogenesis. The data presented in the preceding chapters has provided evidence for a role for germ cells in the regulation of specific Sertoli cell functions.

A theme of this thesis has been the necessity of using an *in vivo* model to study cellular interactions in the adult rat testis instead of the popular *in vitro* culture system utilising cells from immature rats. Isolation of Sertoli cells and the study of their response to hormones and other factors in culture removes the complications due to other factors and the complex cellular interactions in the testis. This technique has been useful for identifying the Sertoli cell response to FSH and testosterone (Rommerts *et al.*, 1978; Means *et al.*, 1980), and has provided direct evidence for specific protein secretion by Sertoli cells (eg. transferrin, Skinner & Griswold, 1980; ABP, Kierszenbaum *et al.*, 1980). Cell culture systems are often used simply because they are easily managed but where possible it is important to confirm data in the whole animal. An *in vitro* system provides information on what the isolated cell type is **capable** of but it cannot be assumed that this will also occur *in vivo*. The close association of Sertoli cells with other cell types in the testis means this is especially true for these cells. Typically, Sertoli cells for investigations in culture are isolated from immature rats of approximately 18-25 days of age and results obtained from experiments using this system have often been extrapolated to the physiological situation in the adult. Some studies have shown that the major proteins secreted by 20 day old and 60 day old animals are similar and this data was assumed to be consistent with similar biochemical responses of Sertoli cells in immature and adult rats (Kissinger *et al.*, 1982). However, a more recent study has shown that the profile of protein secretion from seminiferous tubules isolated either from 28 day old or adult rats was very different (McLaren *et al.*, 1993). The response of Sertoli cells to hormones has also been demonstrated to change with the age of animal from which the cells were isolated (Steinberger *et al.*, 1978). The isolation and culture of Sertoli cells from immature rats therefore appears to provide very artificial conditions for the investigation of cell function. This makes it difficult to draw meaningful conclusions as to how data obtained *in vitro* relates to the normal physiological situation.

The studies described in the present work were all carried out using an *in vivo* model of germ cell depletion from the adult rat testis in an attempt to maintain as near as possible the normal cellular interactions in the testis. This model is useful in that the effects of the toxicant appear to be very specific, only causing damage to pachytene spermatocytes in the testis (Foster, 1983). This enables us to study the effect of depletion of one germ cell type at a time due to the process of maturation depletion. Other *in vivo* experimental models have been used to study the role of germ cells in controlling specific Sertoli cell functions. Local γ-irradiation of the testis destroys spermatogonia but appears to have no other effects (Dym & Clermont, 1970). This in turn causes a specific and selective loss of spermatogenic cells and the effect on Sertoli cell function can be studied. Although both these systems perturb some normal cellular interactions in the testis they appear to be very selective in their action and therefore are preferable to the *in vitro* systems for studying testicular function in the adult.

The majority of studies in this thesis have measured the expression of specific mRNAs by the rat Sertoli cell. The methods used were Northern blot and *in situ* hybridisation which respectively provide a measure of the total amount of a specific mRNA in the testis and demonstrate its cellular localisation. However, the stability of this RNA is not determined nor is it established whether it is being actively transcribed, translated or stored. Therefore it is possible that the changes in total mRNA I have measured due to germ cell depletion may not be due to an increase or a decrease in the rate of transcription but may be attributed to a change in the stability of the mRNA. Active transcription could be measured using nuclear run off assays. However, I was interested in stage specific changes and this would have required microdissection of staged tubules for isolation of nuclei. The amount of dissected staged tubules necessary to isolate enough nuclei for these studies was prohibitive. Therefore nuclear run off assays were not attempted during the course of this study but this aspect would be worth studying in the future.

There is an increasing awareness amongst those who study male reproductive physiology that the testis expresses a great number of mRNAs that have no apparent function there (Ivell, 1992). It would also appear that many of these mRNAs are not translated to produce a functional protein, one example is the 800bp POMC mRNA detected in the testis (Jeannotte *et al.*, 1987). This transcript lacks the 5' non-coding region which contains the site for initiation of translation and the region encoding the signal peptide which targets the peptide precursor into the cells secretory pathway. However, protein products of the POMC gene have been detected in the testis (Tsong *et al.*, 1982). This is most likely due to translation of the full length mRNA which is found in Leydig cells, but at a much lower frequency than the truncated 800bp transcript (Lacaze-Masmonteil *et al.*, 1987). Abundant expression of a non-functional transcript and low levels of expression of the full length, translated mRNA appears to be quite a common finding in the gonads (Ivell, 1992). The detection of hemiferrin mRNA in germ cells and the expression of transferrin mRNA in Sertoli cells at much lower levels may be such a case (see chapter 5). It is not known if hemiferrin is translated although it is associated in low amounts with the polysomal fraction in round spermatids (Stallard *et al.*, 1991). Chapters 4-6 of this thesis reported data from the study of specific Sertoli cell mRNAs, the majority of which are known to be actively translated in the testis to produce functional proteins. Only probes used to study expression of transferrin (chapter 5) and cystatin C (chapter 4) in the testis were observed to detect transcripts which may not be translated. Where possible immunostaining was used to detect the protein product of these mRNAs in testis sections. This was especially important in the study of cystatin C where mRNA was detected in Sertoli cells and the majority of germ cells but the protein was only detected in Sertoli cells and elongate spermatids. The reason for this apparently wasteful expression of mRNA is unknown but it does highlight the necessity for caution when interpreting data obtained by Northern blot analysis or *in situ* hybridisation. A full sequence analysis or nuclease protection assays are necessary to determine if new transcripts detected are likely to be translated to produce a functional protein. Association of the mRNAs with the polysome fraction of the cell may also provide evidence for translation of the mRNA.

This study has shown that germ cells do have the ability to modulate Sertoli cell function although not always at the level of gene expression. One of the many questions which arises from this study is **how** do the germ cells exert their control? This fascinating question has stirred many groups to begin investigations into this aspect of control of testicular function. The majority of groups have concentrated on the isolation of proteins from germ cell conditioned medium which has previously been shown to stimulate a particular aspect of Sertoli cell function *in vitro*. To date several germ cell proteins have been identified as modulators of Sertoli cell function. Sertoli cell transferrin mRNA expression (Stallard & Griswold, 1990) and the secretion of testins, SGP-2 and transferrin (Pineau *et al.*, 1993) have all been shown to be stimulated by proteins present in germ cell conditioned medium. However, these proteins still remain to be fully characterised and their production and distribution *in vivo* to be determined.

Chapter 6 of the present study showed an influence of elongate spermatids on Sertoli cell CP-2 production. As spermatids begin to elongate and condense their nucleus they change from being transcriptionally active to having a transcriptionally inactive nucleus (Hecht, 1990). This is due to the tight packaging of their DNA associated with the replacement of histones with protamines in the nucleus (Ward & Coffey, 1991). Therefore, the influence of elongate spermatids on Sertoli cell function would appear not to be due to changes in specific gene expression which would affect Sertoli cells. However, it is also known that some mRNAs are very stable and can be stored after transcription to be translated at a later date (Kleene *et al.*, 1984). The chromatoid body of spermatids is thought to be a site of storage of these stable RNAs which could be translated in elongate spermatids (Parvinen *et al.*, 1986; Saunders *et al.*, 1992). To date, there is no evidence for this but it remains a possible route by which elongate spermatids could exert their effect on Sertoli cells.

A favoured hypothesis for the effect of elongate spermatids is via their specialised junctions with Sertoli cells which have been described in chapter 2, section 4.1. The movement of elongate spermatids in the seminiferous epithelium from the adluminal area to the base of the Sertoli cells and then back to the lumen for release involves massive restructuring of the Sertoli cell cytoplasm as the spermatids move through it (Russell, 1993a). It has been suggested that the extracellular matrix which is involved in this restructuring could have an influence on gene expression via cytoskeletal connections with the Sertoli cell nuclear transcriptional machinery (Jégou *et al.*, 1992). As yet this remains only a hypothesis and the way forward to provide evidence in support of this theory is unclear. However, it has been shown that secretion of plasminogen activator by immature Sertoli cells in culture is increased when these cells are stimulated to perform phagocytosis (J.A. Grootegoed, personal communication). This protein normally shows the same pattern of secretion by Sertoli cells as does CP-2 with maximal secretion at stages VI and VII of the spermatogenic cycle (Parvinen, 1993). It would be of interest to study the effect of elongate spermatid depletion from the seminiferous epithelium on the level of plasminogen activator mRNA in Sertoli cells. Since phagocytosis involves major restructuring of the Sertoli cell cytoplasm as does spermatid movement within the seminiferous epithelium, this may provide data consistent with the change in elements determining Sertoli cell structure being an important factor in the regulation of the expression of these genes.

In conclusion, the studies described in this thesis have helped towards a fuller understanding of the cellular interactions in the testis. Evidence for germ cell regulation of specific Sertoli cell gene expression has been provided, as was shown by the modulation of CP-2 mRNA levels by elongate spermatids. Also, germ cell control of Sertoli cell function at a post-transcriptional level has been suggested by the failure to show any significant effect of germ cell depletion on ABP mRNA expression where an effect on secretion of this protein has previously been demonstrated (Pineau *et al.*, 1988). Studies in chapter 7 have described a new approach to the investigation of germ cell modulation of Sertoli cell function which may prove useful in future studies. Hopefully, the increase in understanding of cellular interactions in the testis acquired by studies such as those described in the present work will help towards defining the reasons for some cases of male infertility and will perhaps be useful in the design of a form of male contraceptive based on the cellular disruption of spermatogenesis.

Appendix I: Commonly Used Buffers and Agars

Buffers

1 x SSC
0.15M sodium chloride
0.015M sodium citrate
at pH7.0

TE buffer
10mM Tris-HCl
1mM EDTA
at pH8.0

1x TBE
0.089M Tris base
0.089M boric acid
10mM EDTA

SM buffer
10mM Tris, pH7.4
10mM $MgSO_4$
0.01% gelatin

1 x TAE
0.04M Tris base
0.02M sodium acetate
1mM EDTA
at pH7.2

Agars

Luria Bertani (LB) broth
10g bacto-tryptone
5g bacto-yeast extract
10g NaCl
in 1 litre water

LB-agar
LB-broth
with 1.5% bacto-agar

NZY agar
15g agar
5g NaCl
2g $MgSO_4.H_2O$
5g yeast extract
10g NZ amine
in 1 litre water at pH7.0

NZY top agar
7g agarose
5g NaCl
2g $MgSO_4.H_2O$
5g yeast extract
10g NZ amine
in 1 litre water at pH7.0

Bibliography

Abbott, S.D., Docherty, K., Roberts, J.L., Tepper, M.A., Chin, W.W. & Clayton, R.N. (1985). Castration increases luteinising hormone subunit messenger RNA levels in male rat pituitaries. Journal of Endocrinology 107, R1-R4.

Abney, T.O. & Myers, R.B. (1991). 17ß-estradiol inhibition of Leydig cell regeneration in the ethane dimethylsulphonate-treated mature rat. Journal of Andrology **12**, 295-304.

Adams, M.D., Kelley, J.M., Gocayne, J.D., Dubnick, M., Polymeropoulos, M.H., Xiao, H., Merril, C.R., Wu, A., Olde, B., Moreno, R.F., Kerlavage, A.R., McCombie, W.R. & Venter, J.C. (1991). Complemenary DNA sequencing: expressed sequence tags and human genome project. Science **252**, 1651-1655.

Allenby, G. (1990). Methoxyacetic acid. In *Chemical disruption of spermatogenesis*. Ph.D. Thesis, University of Edinburgh. Chapter 6, pp 144-165.

Allenby, G., Foster, P.M.D. & Sharpe, R.M. (1991) Evidence that secretion of immunoactive inhibin by seminifeous tubules from the adult rat testis is regulated by specific germ cell types: correlation between in vivo and in vitro studies Endocrinology 128, 467-476.

Aoki, A. & Fawcett, D.W. (1978). Is there a local feedback from the seminiferous tubules affecting ctivity of the Leydig cells? Biology of Reproduction **19**, 144-158.

Attramadal, A., Bardin, C.W., Gunsalus, G.L., Musto, N.A. & Hansson, V. (1981). Immunocytochemical localisation of androgen binding protein in rat Sertoli cells and epididymal cells. Biology of Reproduction **25**, 983-988.

Bancroft, J.D. & Stevens, A. Eds. (1982). Theory and Practice of Histological Techniques, 2nd Edition. Churchill Livingstone, Edinburgh.

Bardin, C.W., Chen, C-L.C., Morris, P.L., Gerendai, I., Boitani, C., Liotta, A.S., Margioris, A. & Krieger, D.T. (1987). Proopiomelanocortin-derived peptides in testis, ovary, and tissues of reproduction. Recent Progress in Hormone Research **43**, 1-28.

Barnes, B. & Sato, G. (1980). Seum-free cell culture: a unifying approach. Cell **22**, 649-655.

Barrett, A.J. (1987). The cystatins: a new class of peptidase inhibitors. Trends in Biochemical Sciences **12**, 193-196.

Bartlett, J.M.S., Kerr, J.B. & Sharpe, R.M. (1988). The selective removal of pachytene spermatocytes using methoxyacetic acid as an approach to the study *in vivo* of paracrine interactions in the testis. Journal of Andrology **9**, 31-40.

Bartlett, J.M.S., Weinbauer, G.F. & Nieschlag, E. (1989). Differential effects of FSH and testosterone on the maintenance of spermatogenesis in the adult hypophysectomized rat. Journal of Endocrinology **121**, 49-58.

Bartlett, J.M.S., Wu, F.C.W. & Sharpe, R.M. (1987). Enhancement of Leydig cell testosterone secretion by isolated seminiferous tubules during co-perifusion in vitro. Comparison with static co-culture systems. International Journal of Andrology **10**, 603-617.

Belchetz, P.E., Plant, T.M., Nakai, Y., Keogh, E.J. & Knobil, E. (1978). Hypophyseal responses to continuous and intermitant delivery of hypothalamic GnRH. Science **202**, 631-633.

Bellvé, A.R. & Zheng, W. (1989). Growth factors as autocrine and paracrine modulators of male gonadal functions. Journal of Reproduction and Fertility **85**, 771-793.

Benahmed, M., Bernier, M., Ducharme, J.R. & Saez, J.M. (1982). Steroidogenesis of cultured purified pig Leydig cells: secretion and effects of estrogens. Molecular and Cellular Endocrinology **28**, 705-716.

Bergh, A. (1983). Paracrine regulation of Leydig cells by the seminiferous tubules. International Journal of Andrology **6**, 57-65.

Bergh, A. (1985). Development of the stage specific paracrine regulation of Leydig cells by the seminiferous tubules. International Journal of Andrology **8**, 80-85.

Berthold, A.A. (1849). Transplantation of testes. Translation by Raess, B. & Bremner, W. (1981). Historical Aspects of Study of the Testis. In *The Testis* (Eds. H. Burger & D. de Kretser). Raven Press, New York. Chapter 1, pp1-8.

Bhasin, S., Krummen, L.A., Swerdloff, R.S., Morelos, B.S., Kim, W.H., Bizerega, G.S., Ling, N., Esch, F., Shimasaki, S. & Toppari, J. (1989). Stage dependent expresssion of inhibin α and ß-B subunits during the cycle of the rat seminiferous epithelium. Endocrinology **124**, 987-991.

Binder, S., Marchfelder, A., Brennicke, A. & Wissinger, B. (1992). RNA editing in trans-splicing intron sequences of nad2 mRNAs in *Oenothera* mitochondria. Journal of Biological Chemistry **267**, 7615-7623.

Boockfor, F.R. & Schwarz, L.K. (1991). Effects of interleukin-6, interleukin-2, and tumor necrosis factor α on transferrin release from Sertoli cells in culture. Endocrinology **129**, 256-262.

Bortolussi, M., Zanchetta, R., Belvedere, .P. & Colombo, L. (1990). Sertoli and Leydig cell numbers and gonadotropin receptors in rat testis from birth to puberty. Cell and Tissue Research **260**, 185-191.

Bouin, P. & Ancel, P. (1903). Recherches sur les cellules interstitielles der testicule des mammiferes. Arch. Zool. **1**, 437-523.

Bourne, G.A., Regiani, S., Payne, A.H. & Marshall, J.C. (1980). Testicular GnRH receptors - characterization and localization on interstitial tissue. Journal of Clinical Endocrinology and Metabolism **51**, 407-409.

Brown, W.R.A. (1985). Immunohistochemical localisation of the transferrin receptor in the seminiferous epithelium of the rat. Gamete Research **12**, 317-326.

Buttyan, R., Olsson, C.A., Pintar, J., Chang, C., Bandyk, M., Ng, P.-Y. & Sawczuk, I.S. (1989). Induction of the TRPM-2 gene in cells undergoing programmed death. Molecular and Cerllular Biology **9**, 3473-3481.

Buzek, S.W. & Sanborn, B.M. (1988). Increase in testicular androgen receptor during sexual maturation in the rat. Biology of Reproduction **39**, 39-49.

Calkins, J.H., Sigel, M.M., Nankin, H.R. & Lin, T. (1988). Interleukin-1 inhibits Leydig cell steroidogenesis in primary culture. Endocrinology **123**, 1605-1610.

Carlsen, E., Giwercman, A., Keiding, N. & Skakkebaek, N.E. (1992). Evidence for decreasing quality of semen during past 50 years. British Medical Journal **305**, 609-613.

Caron, M.G., Goldstein, S., Savard, K. & Marsh, J.M. (1975). Protein kinase stimulation of a reconstituted cholesterol side chain cleavage enzyme system in bovine corpus luteum. Journal of Biological Chemistry **250**, 5137-5143.

Castellón, E., Janecki, A. & Steinberger, A. (1989). Age-dependent Sertoli cell responsiveness to germ cells *in vitro*. International Journal of Andrology **12**, 439-450.

Catt, K.J., Dufau, M.L. & Tsuruhara, T. (1972). Radioligand-receptor assay of luteinizing hormone and chorionic gonadotropin. Journal of Clinical Endocrinology **34**, 123-132.

Cejka, J. & Fleischman, L.E. (1973). Post-γ-globulin: Isolation and physicochemical characterization. Archives of Biochemistry and Biophysics **157**, 168-176.

Chang, Y.L., Gutell, R., Noller, H.F. & Wool, I.G. (1984). The nucleotide sequence of a rat 18S ribosomal ribonucleic acid gene and a proposal for the secondary structure of 18S ribosomal ribonucleic acid. Journal of Biological Chemistry **259**, 224-230.

Chauhan, S.S., Goldstein, L.J. & Gottesman, M.M. (1991). Expression of cathepsin L in human tumors. Cancer Research **51**, 1478-1481.

Chemes, H.E., Dym, M. & Raj, H.G.M. (1979). Hormonal regulation of Sertoli cell differentiation. Biology of Reproduction **21**, 251-262.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation and acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry **162**, 156-159.

Clayton, D.A. (1991). Nuclear gadgets in mitochondrial DNA replication and transcription. Trends in Biochemical Science **16**, 107-111.

Clayton, R.N., Popkin, R.M. & Fraser, H.M. (1982). Hypothalamic regulation of pituitary gonadotropin-releasing hormone receptors: Effects of gonadotropin-releasing hormone immunoneutralization. Endocrinology **110**, 1116-1123.

Clermont, Y. (1962). Quantitative analysis of spermatogenesis of the rat: a revised model for the renewal of spermatogonia. American Journal of Anatomy **111**, 111-124.

Clermont, Y. & Harvey, S.C. (1965). Duration of the cycle of the seminiferous epithelium in normal, hypophysectomised and hypophysectomised-hormone treated albini rats. Endocrinology **76**, 80-89.

Clermont, Y., Morales, C. & Hermo, L. (1987). Endocytic activities of Sertoli cells in the rat. Annals of the New York Academy of Sciences **513**, 1-15.

Clermont, Y. & Perry, B. (1957). Quantitative study of the cell population of the seminiferous tubules in immature rats. American Journal of Anatomy **100**, 241-267.

Cole, T., Dickson, P.W., Esnard, F., Averill, S., Risbridger, G.P., Gauthier, F. & Schreiber, G. (1989). The cDNA structure and expression analysis of the genes for the cysteine protease inhibitor cysatin C and for β_2-microglobulin in rat brain. European Journal of Biochemistry **186**, 35-42.

Collard, M.W. & Griswold, M.D. (1987) Biosynthesis and molecular cloning of Sulphated Glycoprotein 2 secreted by rat Sertoli cells. Biochemistry **26**, 3297-3303.

Collard, M.W., Sylvester, S.R., Tsuruta, J.K. & Griswold, M.D. (1988) Biosynthesis and molecular cloning of Sulphated Glycoprotein 1 secreted by rat Sertoli cells: Sequence similarity with the 70-kilodalton precursor to Sulphatide/G_{M1} activator. Biochemistry **27**, 4557-4564.

Collin, O., Bergh, A., Damber, J.-E., & Widmark, A. (1993). Control of testicular vasomotion by testosterone and tubular factors in rats. Journal of Reproduction and Fertility **97**, 115-121.

Conn, P.M. (1986). The molecular basis of gonadotropin releasing hormone action. Endocrine Reviews **7**, 3-10.

Cooke, B.A. (1990). Is cyclic AMP an obligatory second messenger for luteinizing hormone? Molecular and Cellular Endocrinology 69, C11-C15.

Cooke, P.S. & Meisami, E. (1991). Early hypothyroidism in rats causes increased adult testis and reproductive organ size but does not change testosterone levels. Endocrinology **129**, 237-243.

Corlu, A., Gérard, N., Rissel, M., Kercret, H., Kneip, B., Guguen-Guillouzo, C. & Jégou, B. (1992). Identification of a plasma membrane protein involved in the cell-cell contact-mediated regulation of the Sertoli cell function. 7th European Workshop on Molecular and Cellular Endocrinology of the Testis, miniposter no.56.

Counis, R. & Jutisz, M. (1991). Regulation of pituitary gonadotropin gene expression. Trends in Endocrinology and Metabolism **2**, 181-187.

Damber, J.E. & Bergh, A. (1992a). Testicular microcirculation-a forgotten essential in andrology ? International Journal of Andrology **15**, 285-292.

Damber, J.E. & Bergh, A. (1992b). Immunohistochemical demonstration of androgen receptors on testicular blood vessels. International Journal of Andrology **15**, 425-434.

Damber, J.E., Maddocks, S., Widmark, A. & Bergh, A. (1992). Testicular blood flow and vasomotion can be maintained by testosterone in Leydig cell-depletd rats. International Journal of Andrology **15**, 385-393.

Danzo, B.J., Pavlou, S.N & Anthony, H.L. (1990). Hormonal regulation of androgen-binding protein in the rat. Endocrinology **127**, 2829-2838.

Darnell, J., Lodish, H. & Baltimore, D. (Eds.) (1986). Assembly of organelles. In *Molecular Cell Biology* Scientific American Books. Chapter 21, pp 923-935.

Darney, K.J. & Ewing, L. (1981). Autoregulation of testosterone secretion in perfused rat testes. Endocrinology **109**, 993-995.

David, K., Dingemanse, E., Freud, J. & Laquer, G. (1935). Ueber krystallinisches mannliches hormon aus hoden (testosteron), wirksamer als aus harn oder aus cholesterin bereites androsteron. Z. Physiol. Chem. **233**, 281-282.

Davis, J.T. & Ong, D.E. (1992). Synthesis and secretion of retinol-binding protein by cultured rat Sertoli cells. Biology of Reproduction **47**, 528-533.

Davis, L.G., Dibner, M.D.& Battey, J.F. Eds. (1986). Basic Methods in Molecular Biology. Elsevier Inc, New York.

de Jong, F.H. (1988). Inhibin. Physiological Reviews **68**, 555-607.

de Kretser, D.M. & Kerr, J.B. (1988). The cytology of the testis. In *The Physiology of Reproduction, Vol. I* (Eds. E.Nobil & J.D.Neill). Raven Press, New York. Chapter 20, pp 837-932.

de Rooij, D.G., van Dissel-Emiliani, F.M.F. & van Pelt, A.M.M. (1989). Regulation of spermatogonial proliferation. Annals of the New York Academy of Sciences **564**, 140-153.

de Winter, J.P., Timmerman, M.A., Vanderstichele, H.M.J., Klaij, I.A., Grootenhuis, A.J., Rommerts, F.F.G. & de Jong, F.H. (1992). Testicular Leydig cells in vitro secrete only inhibin α-subunits, whereas Leydig cell tumors can secrete bioactive inhibin. Moleular and Cellular Endocrinology 83, 105-115.

Djakiew, D. & Dym, M. (1988). Pachytene spermatocytes proteins influence Sertoli cell function. Biology of Reproduction **39**, 11193-1205.

Dorrington, J.H. & Fritz, I.B. (1973). Effects of gonadotropins on cyclic AMP production by isolated seminiferous tubule and interstitial cell preparations. Endocrinology **94**, 395-403.

Dorrington, J.H. & Fritz, I.B. & Armstrong, D.T. (1978). Control of testicular estrogen synthesis. Biology of Reproduction **18**, 55-64.

Dufau, M.L., Sorrell, S.H. & Catt, K.J. (1981). Gonadotropin-induced phosphorylation of endogenous proteins in the Leydig cell. FEBS Letters **131**, 229-234.

Duguid, J.R., Rohwer, R.G. & Seed, B. (1988). Isolation of cDNAs of scrapie-modulated mRNAs by subtractive hybridization of a cDNA library. Proceedings of the National Academy of Sciences, USA **85**, 5738-5742.

Dym, M. & Clermont, Y. (1970). Role of spermatogonia in the repair of the seminiferous epithelium following x-irradiation of the rat testis. The American Journal of Anatomy **128**, 265-282.

Dym, M. & Fawcett, D.W. (1970). The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biology of Reproduction **3**, 308-326.

Dym, M., Raj, G.G.M., Lin, Y.C., Chemes, H.E., Kotite, N.J., Nayfeh, S.N. & French, F.S. (1979). Is FSH required for maintenance of spermatogenesis in adult rats? Journal of Reproduction and Fertility Supplement **26**, 175-181.

Ellis, G.B. & Desjardins, C. (1982). Male rats secrete luteinizing hormone and testosterone episodically. Endocrinology **110**, 1618-1627.

Ellis, G.B., Desjardins, C. & Fraser, H.M. (1983). Control of pulsatile LH release in male rats. Neuroendocrinolgy **37**, 177-183.

Enders, G. (1993). Sertoli-Sertoli and Sertoli-germ cell communications. In *The Sertoli Cell* (Eds. L.D. Russell & M.D.Griswold) Cache River Press, Florida. Chapter 19, pp 447-460.

Erickson-Lawrence, M., Zabludoff, S.D. & Wright,W.W. (1991) Cyclic Protein 2, a secretory product of rat Sertoli cells, is the proenzyme form of cathepsin L. Molecular Endocrinology **5**, 1789-1798.

Esnard, A., Esnard, F., Guillou, F. & Gauthier, F. (1992). Production of the cysteine protease inhibitor cystain C by rat Sertoli cells. FEBS Letters **300**, 131-135.

Fabbri, A., Knox, G., Buczko, E. & Dufau, M.L. (1988). ß-endorphin production by the fetal leydig cell: regulation and implications for paracrine control of Sertoli cell function. Endocrinology **122**, 749-755.

Fakunding, J.K., Tindall, D.J., Dedman, J.R., Mena, C.R. & Means, A.R. (1976). Biochemical actions of follicle stimulating hormone in the Sertoli cell of the rat testis. Endocrinology **98**, 392-402.

Fauser, B.C., Baird, A. & Hsueh, A.J. (1988). Fibroblast growth factor inhibits luteinizing hormone-stimulated androgen production by cultured rat testicular cells. Endocrinology **123**, 2935-2941.

Fawcett, D.E., Leak, K.V. & Heidger, P.M., Jr. (1970). Electron microscopic observations on the structural components of the blood-testis barrier. Journal of Reproduction and Fertility (Supplement) **10**, 105-122.

Feinberg, A.P., Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry **132**, 6-13.

Felden, F., Guéant, J.L., Ennya, A., Gérard, A., Frémont, S., Nicolas, J.P. & Gérard, H. (1992). Photoaffinity labelled rat androgen-binding protein and human sex hormone steroid-binding protein bind specifically to rat germ cells. Journal of Molecular Endocrinology **9**, 39-46.

Foster, P.M.D., Lloyd, S.C. & Blackburn, D.M. (1987). Comparison of the in vivo and in vitro testicular effects produced by methoxy-, ethoxy-, and N-butoxy acetic acids in the rat. Toxicology **43**, 17-30.

Foster, P.M.D., Creasy, D.M., Foster, J.R., Thomas, L.V., Cook, M. & Gangolli, S.D. (1983). Testicular toxicity of ethylene glycol monomethyl and monoethyl ethers in the rat. Toxicology and Applied Pharmacology **69**, 385-399.

Fouquet, J.P. (1987). Ultrastructural analysis of a local regulation of Leydig cells in the adult monkey (*Macaca fascicularis*) and rat. Journal of Reproduction and Fertility **79**, 49-56.

Freeman, D.A. & Ascoli, M. (1981). Desensitization to gonadotropins in cultured Leydig tumour cells involves loss of gonadotropin receptors and decresed capacity for steroidogenesis. Proceedings of the National Academy of Sciences USA **78**, 6309-6313.

French, F.S. & Ritzen, E.M. (1973). A high-affinity androgen binding protein (ABP) in rat testis: evidence for secretion into efferent duct and adsorption by epididymis. Endocrinology **93**, 88-95.

Fritz, I.B., Burdzy, K., Setchell, B. & Blaschuk, O. (1983). Ram rete testis fluid contains a protein (clusterin) which influences cell-cell interactions in vitro. Biology of Reproduction **28**, 1173-1188.

Fritz, I.B., Tung, P.S. & Ailenberg, M. (1993). Proteases and antiproteases in the seminiferous tubule. In *The Sertoli Cell* (Eds. L.D. Russell & M.D.Griswold) Cache River Press, Florida. Chapter 9, pp 217-235.

Gal, S. & Gottesman, M.M. (1986).The major excreted protein of transformed fibroblasts is an activable acid-protease. Journal of Biological Chemistry **261**, 1760-1765.

Galdieri, M., Monaco, L. & Stefanini, M. (1984). Secretion of Androgen Binding Protein by Sertoli cells is influenced by contact with germ cells. Journal of Andrology **5**, 409-415.

Gangnerau, M.-N., Funkenstein, B. & Picon, R. (1982). LH/hCG receptors and stimulation of testosterone biosynthesis in the rat testis: changes during foetal development in vivo and in vitro. Molecular and Cellular Endocrinolgy **28**, 499-512.

Gérard, A., Ennya, A., Egloff, M., Domingo, M., Degrelle, H & Gérard, H. (1992). Endocytosis of human sex steroid-binding protein in monkey germ cells. Annals of the New York Academy of Sciences **637**, 258-277.

Gérard, N., Syed, V., Bardin, W., Genetet, N. & Jégou, B. (1991). Sertoli cells are the site of interleukin-1α synthesis in rat testis. Molecular and Cellular Endocrinology **82**, R13-R16.

Gérard, N., Syed, & Jégou, B. (1992). The Sertoli cell IL-1 production is activated by lipopolysaccharides, latex beads and residual bodies. 7th European Workshop on Molecular and Cellular Endocrinology of the Testis, miniposter no.111.

Geremia, R., D'Agostino, A. & Monesi, V. (1978). Biochemical evidence of haploid gene activity in spermatogenesis of the mouse. Experimental Cell Research **111**, 23-30.

Gerendai, I., Shaha, C., Thau, R. & Bardin, C.W. (1984). Do testicular opiates regulate Leydig cell function? Endocrinology **115**, 1645-1647.

Gharib, S.D., Wierman, M.E., Badger, T.M. & Chin, W.W. (1989). Sex steroid hormone regulation of follicle-stimulating hormone subunit messenger ribonucleic acid (mRNA) levels in the rat. Journal of Clinical Investigation **80**, 294-299.

Giwercman, A. & Skakkebaek, N.E. (1992). The human testis - an organ at risk? International Journal of Andrology **15**, 373-375.

Gizang-Ginsberg, E. & Wolgemuth, D.J. (1985). Localiation of mRNAs in mouse testis by in situ hybridisation: distribution of α-tubulin and developmental stage specificity of pro-opiomelanocortin transcripts. Developmental Biology **111**, 293-305.

Godine, J.E., Chin, W.W. & Habener, J.F. (1980). Luteinizing and follicle stimulating hormones: cell-free translations of mRNAs coding for subunit precursors. Journal of Biological Chemistry **255**, 8780-8783.

Gower, D.B. (1988). The biosynthesis of steroid hormones: an update. In *Hormones and their Actions, Part I.* (Eds. B.A. Cooke, R.J.B. King & H.J. van der Molen). Elsevier Science Publishers. Chapter 1, pp 3-28.

Greep, R.O., Fevold, H.L. & Hisaw, F.L. (1936). Effects of two hypophyseal gonadotropic hormones on the reproductive system of the male rat. Anatomical Record **65**; 261-271.

Grima, J., Pineau, C., Bardin, C.W. & Cheng, C.Y. (1992) Rat Sertoli cell clusterin, α_2-macroglobulin, and testins: biosynthesis and differential regulation by germ cells. Molecular and Cellular Endocrinology **89**, 127-140.

Grino, P.B., Griffin, J.E. & Wilson, J.D. (1990). Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone. Endocrinology **126**, 1165-1172.

Griswold, M.D., Solari, A., Tung, P.S. & Fritz, I.B. (1977). Stimulation by follicle-stimulating hormone of DNA synthesis and of mitosis in cultured Sertoli cells prepared from testes of immature rats. Molecular and Cellular Endocrinology **7**, 151-165.

Grootegoed, J.A., Peters, M.J., Mulder, E., Rommerts, F.F.G. & van der Molen, H.J. (1977). Absence of a nuclear androgen receptor in isolated germinal cells of rat testis. Molecular and Cellular Endocrinology **9**, 159-167.

Haisenleder, D.J., Katt, J.A., Ortolano, G.A., El-Gewely, M.R.,Duncan, J.A., Dee, C & Marshall, J.C. (1988). Influence of gonadotropin releasing hormone pulse amplitude, frequency and treatment duration on the regulation of luteinizing hormone

(LH) subunit messenger ribonucleic acids and LH secretion. Molecular Endocrinology **2**, 338-343.

Hall, P.F., Irby, D.C. & de Kretser, D.M. (1969). Conversion of cholesterol to androgens by rat testes: comparison of interstitial cells and seminiferous tubules. Endocrinology **84**, 488-496.

Hall, S.H., Conti, M., French, F.S. & Joseph, D.R. (1990). Follicle-stimulating hormone regulation of androgen-binding protein messenger RNA in Sertoli cell cultures. Molecular Endocrinology **4**, 349-355.

Hamernik, D.L., Crowder, M.E., Nilson, J.H. & Nett, T.M. (1986). Measurement of messenger ribonucleic acid for luteinizing hormone ß-subunit, α–subunit, growth hormone, and prolactin gonadotropins after hypothalamic-pituitary disconnection in ovariectomized ewes. Endocrinology **119**, 2704-2710.

Hammond, G.L., Underhill, D.A., Rykse, H.M. & Smith, C.L. (1989). The human sex hormone-binding globulin gene contains exons for androgen-binding protein and two other testicular messenger RNAs. Molecular Endocrinology **3**, 1869-1876.

Hardy, M.P., Zirkin, B.R. & Ewing, L.L. (1989). Kinetic studies on the development of the adult population of Leydig cells in testes of the pubertal rat. Endocrinology **124**, 762-770.

Hecht, N.B. (1990). Regulation of 'haploid expressed genes' in male germ cells. Journal of Reproduction and Fertility **88**, 679-693.

Hecht, N.B. & Kennington, E. (1983). Temporal synthesis of mitochondrial proteins from mouse epididymal spermatozoa. Gamete Research **7**, 289-297.

Heckert, L.L. & Griswold, M.D. (1991). Expression of follicle-stimulating hormone receptor mRNA in rat testes and Sertoli cells. Molecular Endocrinology **5**, 670-677.

Heideran, M.A. & Kistler, W.S. (1987). Isolation of a cDNA clone for transition protein 1 (TP1), a major chromosomal protein of mammalian spermatids. Gene **54**, 281-284.

Hedrick, S.M., Cohen, D.I., Nielsen, E.A. & Davis, M.M. (1984). Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. Nature **308**, 149-153.

Hermo. L., Wight, J., Oko, R. & Morales, C.R. (1991). Role of epithelial cells of the male excurrent duct system of the rat in the endocytosis or secretion of sulphated glycoprotein-2 (clusterin). Biology of Reproduction **44**, 1113-1131.

Hess, R.A., Cooke, P.S., Bunick, D. & Kirby, J.D. (1993). Adult testicular enlargement induced by neonatal hypothyroidism is accompanied by increased Sertoli and germ cell numbers. Endocrinology **132**, 2607-2613.

Hess, R.A., Schaeffer, D.J., Eroschenko, V.P. & Keen, J.E. (1990). Frequency of stages in the cycle of the seminiferous epithelium. Biology of Reproduction **43**, 517-524.

Holmes, S.D., Bucci, L.R., Lipschultz, L.I. & Smith, R.G. (1983). Transferrin binds specifically to pachytene spermatocytes. Endocrinology **113**, 1916-1918.

Howell, J.McC., Thompson, J.N. & Pitt, G.A.J. (1963). Histology of the lesions produced in the reproductive tract of animals fed a diet deficient in vitamin A alcohol but containing vitamin A acid. Jornal of Reproduction and Fertility **5**, 159-167.

Hsueh, A.J.W., Dahl, K.D., Vaughan, J., Tucker, E., Rivier, J., Bardin, C.W. & Vale, W.W. (1987). Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. Proceedings of the National Academy of Sciences, USA **84**, 5082-5086.

Huggenvik, J.I., Idzerda, R.L., Haywood, L., Lee, D.C., McKnight, G.S. & Griswold, M.D. (1987). Transferrin messenger ribonucleic acid: molecular cloning and hormonal regulation in rat Sertoli cells. Endocrinology **120**, 332-340.

Huggenvik, J.I., Sylvester, S.R. & Griswold, M.D. (1984). Control of mRNA synthesis in Sertoli cells. Annals of the New York Academy of Sciences **438**, 1-7.

Hugly, S. Roberts, K. & Griswold, M.D. (1988). Transferrin and sulphated glycoprotein-2 messenger ribonucleic acid levels in the testis and isolated Sertoli cells of hypophysectomised rats. Endocrinology **122**, 1390-1396.

Huhtaniemi, I.T., Nevo, N., Amsterdam., A. & Naor, Z. (1986). Effect of postnatal treatment with a gonadotropin-releasing hormone antagonist on sexual maturation of male rats. Biology of Reproduction **35**, 501-509.

Ibrahim, S.N. Moussa, S.M. & Childs, G.V. (1986). Morphometric studies of rat anterior pituitary cells after gonadectomy: correlation of changes in gonadotropes with the serum levels of gonadotropins. Endocrinology **119**, 629-637.

Isomaa, V., Parvinen, M., Janne, O.A. & Bardin, C.W. (1985). Nuclear androgen receptors in different stages of the seminiferous epithelial cycle and the nterstitial tissue of rat testis. Endocrinology **116**, 132-137.

Ivell, R. (1992). "All that glisters is not gold": common testis gene transcripts are not always what they seem. International Journal of Andrology **15**, 85-92.

Jeannotte, L., Burbach, J.P.H. & Drouin, J. (1987). Unusual proopiomelanocortin ribonucleic acids in extrapituiary tissues: intronless transcripts in testes and long poly (A) tails in hypothalamus. Molecular Endocrinology **1**, 749-757.

Jégou, B. (1991). Spermatids are regulators of Sertoli cell funtion. Annals of the New York Acadamy of Science **637**, 340-353.

Jégou, B. (1992). The Sertoli cell. Ballieres Clinical Endocrinology and Metabolism **6**, 273-311.

Jégou, B., Laws, A.O. & De Kretser, D.M. (1984). Changes in testicular function induced by short-term exposure of rat to heat: further evidence for interaction of germ cells, Sertoli cells and Leydig cells. International Journal of Andrology **7**, 244-257.

Jégou, B., Le Gac, F. & de Kretser, D.M. (1982). Seminiferous tubule fluid and interstitial fluid production. I. Effects of age and hormonal regulation in immature rats. Biology of Reproduction **27**, 590-595.

Jégou, B., Le Gac, F., Irby, D.C. & de Kretser, D.M. (1983). Studies on seminiferous tubule fluid production in the adult rat: effect of hypophysectomy and treatment with FSH, LH and testosterone. International Journal of Andrology **6**, 249-260.

Jégou, B., Pineau, C., Velez de la Calle, J.P., Touzalin, A-M., Bardin, C.W. & Cheng, C.Y. (1993). Germ cell control of testin production is inverse to that of other Sertoli cell products. Endocrinology **132**, 2557-2562.

Jégou, B., Syed, V., Sourdaine, P., Byers, S., Gérard, N., Velez de la Calle, J., Pineau, C., Garnier, D.H. & Bauché, F. (1992). The dialogue between late spermatids and Sertoli cells in vertebrates: a century of research. In *Spermatogenesis, Fertilization, Contraception: Molecular, Cellular and Endocrine Events in Male Reproduction* (Eds. E. Nieschlag & U.-F. Habenicht).Springer-Verlag, Berlin. pp 57-95.

Johnson, D.A., Barrett, A.J. & Mason, R.W. (1986). Cathepsin L inactivates α_1-proteinase inhibitor by cleavage in the reactive site region. Journal of Biological Chemistry **261**, 14748-14751.

Joseph, D.R., Hall, S.H. & French, F.S. (1985). Identification of complementary DNA clones that encode rat androgen binding protein. Journal of Andrology **6**, 392-395.

Joseph, D.R., Hall, S.H. & French, F.S. (1987). Rat androgen binding protein: evidence for identical subunits and amino acid sequence homology with human sex hormone-binding globulin. Proceedings of the National Academy of Sciences **84**, 339-343.

Joseph, D.R., Lawrence, W. & Danzo, B.J. (1992). The role of asparagine-linked oligosaccharides in the subunit structure, steroid binding, and secretion of androgen-binding protein. Molecular Endocrinology **6**, 1127-1134.

Jutte, N.H.P.M., Jansen, R., Grootegoed, J.A., Rommerts, F.F.G., Clausen, O.P.F. & van der Molen, H.J. (1982). Regulation of survival of rat pachytene spermatocytes by lactate supply by Sertoli cells. Journal of Reproduction and Fertility **65**, 431-438.

Kaipia, A., Parvinen, M., Shimasaki, S., Ling, N. & Toppari, J. (1991). Stage-specific cellular regulation of inhibin α-subunit mRNA expression in the rat seminiferous epithelium. Molecular and Cellular Endocrinology **82**, 165-173.

Kangasniemi, M., Cheng, C.Y., Toppari, J., Grima, J., Stahler, M., Bardin, C.W. & Parvinen, M. (1992). Basal and FSH-stimulated steady state levels of SGP-2, α_2-macroglobulin, and testibumin in culture media of rat seminiferous tubules at defined stages of the epithelial cycle. Journal of Andrology **13**, 208-213.

Karzai, A.W. & Wright, W.W. (1992). Regulation of the synthesis and secretion of transferrin and cyclic protein-2/cathepsin L by mature rat Sertoli cells in culture. Biology of Reproduction **47**, 823-831.

Kerr, J.B., Maddocks, S. & Sharpe, R.M. (1992). Testosterone and FSH have independent, synergistic and stage-dependent effects upon spermatogenesis in the rat testis. Cell and Tissue Research **268**, 179-189.

Kerr, J.B., Millar, M., Maddocks, S. & Sharpe, R.M. (1993). Stage-dependent changes in spermatogenesis and Sertoli cells in relation to the onset of spermatogenic failure following withdrawal of testosterone. The Anatomical Record **235**, 547-559.

Kerr, J.B. & Sharpe, R.M. (1985). Follicle-stimulating hormone induction of Leydig cell maturation. Endocrinology **116**, 2592-2604.

Khan, S., Teerds, K. & Dorrington, J. (1992). Growth factor requirements for DNA synthesis by Leydig cells from the immature rat. Biology of Reproduction **46**, 335-341.

Kierszenbaum, A.L., Feldman, M., Lea, O., Spruill, W.A., Tres, L.L., Petrusz, P. & French, F.S. (1980). Localisation of androgen-binding protein in proliferating Sertoli cells in culture. Biology of Reproduction **27**, 233-240.

Kim, K.H. & Griswold, M.D. (1990). The regulation of retinoic acid receptor mRNA levels during spermatogenesis. Molecular Endocrinology **4**, 1679-1688.

Kirschke, H., Kembhavi, A.A., Bohley, P. & Barrett, A.J. (1982). Action of rat liver cathepsin L on collagen and other substrates. Biochemical Journal **201**, 367-372.

Kirszbaum, L., Sharpe, J.A., Murphy, B., d'Apice, A.J.F., Classon, B., Hudson, P. & Walker, I.D. (1988). Molecular cloning and characterization of the novel human complement-associated protein, SP-40,40: a link between the complement and reproductive systems. EMBO Journal **8**, 711-718.

Kissinger, C., Skinner, M.K. & Griswold, M.D. (1982). Analysis of Sertoli cell-secreted proteins by two-dimensional gel electrophoresis. Biology of Reproduction **27**, 233-240.

Kleene, K.C., Distel, R.J. & Hecht, N.B. (1983). cDNA clones encoding cytoplasmic poly $(A)^+$ RNAs which first appear at detectable levels in haploid phases of spermatogenesis in the mouse. Developmental Biology **98**, 455-464.

Kleene, K.C. & Flynn, J.F. (1987). Characterisation of a cDNA clone encoding a basic protein TP2, involved in chromatin condensation in the mouse. Journal of Biological Chemistry **262**, 17272-17277.

Kliesch, S., Penttilä, T.-L., Gromoll, J., Saunders, P.T.K., Nieschlag, E. & Parvinen, M. (1992). FSH receptor mRNA is expressed stage dependently during rat spermatogenesis. Molecular and Cellular Endocrinology **84**, R45-R49.

Ko, M.S., Ko, S.B., Takahashi N., Nishiguchi K.& Abe K. (1990). Unbiased amplification of a highly complex mixture of DNA fragments by 'lone-linker'-tagged PCR. Nucleic Acids Research **18**, 4293-4294.

Krummen, L.A., Toppari, J., Kim, W.H., Morelos, B.S., Ahmad, N., Swerdloff, R.S., Ling, N., Shimasaki, S., Esch, F., & Bhasin, S. (1989). Regulation of testicular inhibin subunit messenger ribonucleic acid levels in vivo: effects of hypophysectomy and selective follicle-stimulating hormone replacement. Endocrinology **125**, 1630-1637.

Krummen, L.A., Wong, W.L., Garg, S., Gibson, U., Mann, E. & Mather, J.P. (1992). Activin A is produced in vitro by interstitial cells derived from immature, but not adult rat testis. VII European Testis Workshop on Molecular and Cellular Endocrinology of the Testis. Abstract book, p 76.

Ku, C.Y., Lu., Q., Ussuf., K.K., Weinstock, G.M. & Sanborn, B.M. (1991). Hormonal regulation of cytochrome oxidase subunit nessenger RNAs in rat Sertoli cells. Molecular Endocrinology **5**, 1669-1676.

Kumar, T.R. & Low, M.J. (1993). Gonadal steroid hormonal regulation of human and mouse follicle stimulating hormone ß-subunit gene expression in vivo. Molecular Endocrinology **7**, 898-906.

Lacaze-Masmonteil, T., de Keyzer, Y., Lutan, J.-P., Kahn, A. & Bertagna, X. (1987). Characterization of proopiomelanocortin transcripts in human non-pituitary tissues. Proceedings of the National Academy of Sciences, USA **84**, 7261-7265.

Laskey, J., Webb, I., Schulman, H.M. & Ponka, P. (1988). Evidence that transferrin supports cell proliferation by supplying iron for DNA synthesis. Experimental Cell Research **176**, 87-95.

Leblond, C.P. & Clermont, Y. (1952).Definition of the stages of the cycle of the seminiferous epithelium in the rat. Annals of the New York Acadamy of Science **55**, 548-573.

Lee, N.T., Chae, C-B. & Kierszenbaum, A.L. (1986). Contrasting levels of transferrin gene activity in cultured rat Sertoli cells and intact seminiferous tubules. Proclamations of the National Acadamy of Science, USA **83**, 8177-8181.

Le Magueresse, B. & Jégou, B. (1988a) Paracrine control of immature Sertoli cells by adult germ cell, in the rat (an *in vitro* study). Cell-cell interactions in the testis. Molecular and Cellular Endocrinology **58**, 65-72.

Le Magueresse, B. & Jégou, B. (1988b). *In vitro* effects of germ cells on the secretory activity of Sertoli cells recovered from rats of different ages. Endocrinology **122**, 1672-1680.

Le Magueresse, B., Le Gac, F., Loir, M. & Jégou, B. (1986). Stimulation of rat Sertoli cell secretory activity *in vitro* by germ cells and residual bodies. Journal of Reproduction and Fertility **77**, 489-498.

Le Magueresse, B., Pineau, C., Guillou, F. & Jégou, B. (1988). Influence of germ cells upon transferrin secretion by rat Sertoli cells *in vitro*. Journal of Endocrinology **118**, R13-R16.

Lin, T., Calkins, H., Morris, P.L., Vale, W.W. & Bardin, C.W. (1989). Regulation of Leydig cell function in primary culture by inhibin and activin. Endocrinology **125**, 2134-2140.

Lin, T., Calkins, H., Morris, P.L., Vale, W.W. & Bardin, C.W. (1989). Regulation of Leydig cell function in primary culture by inhibin and activin. Endocrinology **125**, 2134-2140.

Linder, C., Heckert, L., Roberts, K., Kim, K.H. & Griswold, M.D. (1991). Expression of receptors during the cycle of the seminiferous epithelium. Annals of the New York Academy of Sciences **637**, 313-321.

Ling, N., Ying, S-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. & Guillemin, R. (1986a). Pituitary FSH is released by a heterodimer of the ß subunits from the two forms of inhibin. Nature **321**, 779-782.

Ling, N., Ying, S-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. & Guillemin, R. (1986b). A homodimer of the ß subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. Biochemical and Biophysical Research Communications **138**, 1129-1137.

Loros, J.J., Denome, S.A. & Dunlap, J.C. (1989). Molecular cloning of genes under control of the circadian clock in *Neurospora*. Science **243**, 385-388.

McCormack, J.T., Plant, T.M., Hess, D.L. & Knobil, E. (1977). The effect of luteinizing hormone releasing hormone (LHRH) antiserum administration on gonadotropin secretion in the rhesus monkey. Endocrinology **100**, 663-667.

McFarland, K.C., Sprengel, R., Phillips, H.S., Köhler, M., Rosemblit, N., Nikolics, K., Segaloff, D.L. & Seeburg, P.H. (1989). Lutropin-choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family. Science **245**, 494-499.

McGinlay, D.M., Posalaky, Z., Porvaznik, M. & Russell, L. (1979). Gap junctions between Sertoli and germ cells of rat seminiferous tubules. Tissue and Cell **11**, 741-754.

McKinnell, C. & Sharpe, R.M. (1992). The role of specific germ cell types in modulation of secretion of androgen-regulated proteins (ARPs) by stage VI-VIII seminiferous tubules from the adult rat. Molecular and Cellular Endocrinology **83**, 219-231.

McLachlan, R.I., Dahl, K.D., Bremner, W.J., Schwall, R., Schmelzer, C.H., Mason, A.J. & Steiner, R.A. (1989). Recombinant human activin-A stimulates basal FSH and GnRH-stimulated FSH and LH release in the adult male macaque, *Macaca fascicularis*. Endocrinology **125**, 2787-2789.

McLaren, T.T., Foster, P.M.D. & Sharpe, R.M. (1993). Effect of age on seminiferous tubule protein secretion and the adverse effects of testicular toxicants in the rat. International Journal of Andrology **16**, 370-379.

Mali, P., Kaipia, A., Kangasniemi, M., Toppari, J., Sandberg, M., Hecht, N. & Parvinen, M. (1989). Stage-specific expression of nucleoprotein mRNAs during rat and mouse spermiopgenesis. Reproduction, Fertility and Development **1**, 369-382.

Marian, J. & Conn, P.M. (1979). GnRH stimulation of cultured pituitary cells requires calcium. Molecular Pharmacology **16**, 196-201.

Mason, R.W., Johnson, B.A., Barrett, A.J. & Chapman, H.A. (1986). Elastinolytic activity of human cathepsin L. Biochemical Journal **233**, 925-927.

Mather, J.P., Gunsalus, G.L., Musto, N.A., Cheng, C.Y., Parvinen, M., Wright, W., Perez-Infante, V., Margioris, A., Liotta, A., Becker, R., Krieger, D.T. & Bardin, C.W. (1983). The hormonal and cellular control of Sertoli cell secretion. Journal of Steroid Biochemistry **19**, 499-504.

Mather, J.P. & Krummen, L.A. (1992). Inhibin, activin and growth factors: paracrine regulators of testicular function. In *Spermatogenesis, Fertilization, Contraception: Molecular, Cellular and Endocrine Events in Male Reproduction* (Eds. E. Nieschlag & U.-F. Habenicht).Springer-Verlag, Berlin. pp 169-200.

Matsumoto, A.M. & Bremner, W.J. (1984). Modulation of pulsatile gonadotropin secretion by testosterone in man. Journal of Clinical Endocrinology and Metabolism **58**, 609-614.

Matsumoto, A.M., Karpas, A.E. & Bremner, W.J. (1986). Chronic human chorionic gonadotropin administration in normal men: evidence that follicle-stimulating hormone is necessary for the maintenance of quantitatively normal spermatogenesis in man. Journal of Clinical Endocrinology and Metabolism **62**, 1184-1192.

Means, A.R., Dedman, J.R., Tash, J.S., Tindall, D.J., van Sickle, M. & Welsh, M.J. (1980). Regulation of the testis Sertoli cell by follicle stimulating hormone. Annual Reviews of Physiology **42**, 59-70.

Meistrich, M.L., Longtin, J., Brock, W.A., Grimes, S.R. & Mace, M.L. (1981). Purification of rat spermatogenic cells and preliminary biochemical analysis of these cells. Biology of Reproduction **25**, 1065-1077.

Mercer, J.E., Clements, J.A., Funder, J.W. & Clarke, I.J. (1987). Rapid and specific lowering of pituitary FSHß mRNA levels by inhibin. Molecular and Cellular Endocrinology **53**, 251-254.

Millar, M.R., Sharpe, R.M., Maguire, S.M. & Saunders, P.T.K. (1993). Cellular Localisation of Messenger RNAs in Rat Testis: Application of Digoxigenin-labelled Ribonucleotide Probes to Embedded Tissue. Cell and Tissue Research **273**, 269-277.

Millar, M.R., Sharpe, R.M., Maguire, S.M., Gaughan, J., West, A.P. & Saunders, P.T.K. (1994). Localisation of specific mRNA's by in situ hybridisation to the residual body at stages IX-X of the cycle of the rat seminiferous epithelium: fact or artefact? Biology of Reproduction. Submitted.

Monesi, V., Geremia, R., D'Agostino, A. & Boitani, C. (1978). Biochemistry of male germ cell differentiation in mammals: RNA synthesis in meiotic and postmeiotic cells. In *Current Topics in Developmental Biology Volume 12*. Chapter 2, pp11-36.

Morales, C.R., Alcivar, A.A., Hecht, N.B. & Griswold, M.D. (1989). Specific mRNAs in Sertoli and germinal cells of testes from stage synchronized rats. Molecular Endocrinology **3**, 725-733.

Morales, C. & Clermont, Y. (1993). Structural changes of the Sertoli cell during the cycle of the seminiferous epithelium. In *The Sertoli Cell* (Eds. L.D. Russell and M.D.Griswold) Cache River Press, Florida. Chapter 13, pp 305-329.

Morales, C. Clermont, Y. & Hermo, L. (1985). Nature and function of endocytosis in Sertoli cells of the rat. The American Journal of Anatomy **173**, 203-217.

Morales, C., Hugly, S. & Griswold, M.D. (1987). Stage-dependent levels of specific mRNA transcripts in Sertoli cells. Biology of Reproduction **36**, 1035-1046.

Moyle, W.R. & Ramachandran, J. (1973). Effect of LH on steroidogenesis and cyclic AMP accumulation in rat leydig cell preparations and mouse tumor leydig cells. Endocrinology **93**, 127-134.

Namiki, M., Yokokawa, K., Okuyama, A., Koh, E., Kiyohara, H., Nakao, M., Sakoda, S., Matsumoto, K. & Sonoda, T. (1991). Evidence for thepresence of androgen receptors in human Leydig cells. Journal of Steroid Biochemistry and Molecular Biology **38**, 79-82.

Niemi, M., Sharpe, R.M. & Brown, W.R.A. (1986). Macrophages in the interstitial tissue of the rat testis. Cell and Tissue Research **243**, 337-344.

Nilsen-Hamilton, M., Hamilton, R.T., Allen, W.R. & Massoglia, S.L. (1981).Stimulation of the release of two glycoproteins from mouse 3T3 cells by growth factors and by agents that increase intralysomal pH. Biochemical and Biophysical Research Communications **101**, 411-417.

Norton, J.N. & Skinner, M.K. (1989). Regulation of Sertoli cell function and differentiation through the actions of a testicular paracrine factor P-Mod-S. Endocrinology **124**, 2711-2719.

O'Brien, J.S., Kretz, K.A., Dewji, N., Wenger, D.A., Esch, F. & Fluharty, A.L. (1988). Coding of two sphingolipid activator proteins (SAP-1 and SAP-2) by same genetic locus. Science **241**, 1098-1101.

Ogawa, K., Kurohmaru, M., Shiota,K., Takahashi, M., Nishida, T. & Hayashi, Y. (1991). Histochemical localization of inhibin and activin α, ßA and ßB subunits in rat gonads. Journal of Veterinary Medical Science **53**, 207-212.

Okayama, H. & Berg, P. (1982). High efficiency cloning of full-length cDNA. Molecular and Cellular Biology **2**, 161-170.

Onoda, M. & Djakiew, D.(1990). Modulation of Sertoli cell secretory function by rat round spermatid protein(s). Molecular and Cellular Endocrinology **73**, 35-44.

Onoda, M., Suarez-Quian, C.A., Djakiew, D. & Dym, M. (1990). Characterisation of Sertoli cells cultured in the bicameral chamber system: relationship between formation of permeability barriers and polaried secretion of transferrin. Biology of Reproduction **43**, 672-683.

Onoda, M., Djakiew, D. & Papadopoulos, V. (1991). Pachytene spermatocytes regulate the secretion of Sertoli cell protein(s) which stimulate Leydig cell steroidogenesis. Molecular and Cellular Endocrinology **77**, 207-216.

Oonk, R.B., Jansen, R. & Grootegoed, J.A. (1989). Differential effects of follicle-stimulating hormone, insulin, and insulin-like growth factor I on hexose uptake and lactate production by rat Sertoli cells. Journal of Cellular Physiology **139**, 210-218.

Orth, J.M. (1982). Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. Anatomical Record **203**, 485-492.

Orth, J.M. (1984). The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. Endocrinology **115**, 1248-1255.

Orth, J.M. (1986). FSH-induced Sertoli cell proliferation in the developing rat is modified by ß-endorphin produced in the testis. Endocrinology **119**, 1876-1878.

Orth, J.M. & Christensen, A.K. (1978). Autoradiographic localization of specifically bound ^{125}I-labelled follicle stimulating hormone on spermatogonia of the rat testis. Endocrinology **103**, 1944-1951.

Orth, J.M., Gunsalus, G.L. & Lamperti, A.A. (1988). Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. Endocrinology **122**, 787-794.

Orth, J.M., Higginbotham, C.A. & Salisbury, R.L. (1984). Hemicastration causes and testosterone prevents enhanced uptake of [^{3}H] thymidine by Sertoli cells in testes in immature rats. Biology of Reproduction **30**, 263-270.

Osterlind, A. (1986). Diverging trends in incidence and mortality of testicular cancer in Denmark, 1943-1982. British Journal of Cancer **53**, 501-505.

Paniagua, R., Codesal, J., Nistal, M., Rodriguez, M.C. & Santamaria, L. (1987). Quantification of cell types throughout the cycle of the human seminiferous epithelium and their DNA content. Anatomy and Embryology **176**, 225-230.

Papadopoulos, V. (1991). Identification and purification of a human Sertoli cell-secreted protein (hSCSP-80) stimulating Leydig cell steroid biosynthesis. Journal of Clinical Endocrinology and Metabolism **72**, 1332-1339.

Papadopoulos, V., Kamtchouing, P., Drodowsky, M.A., Hochereau de Reviers, M.T. & Carreau, S. (1987). Adult rat Sertoli cells secrete a factor or factors which modulate Leydig cell function. Journal of Endocrinology **114**, 459-467.

Parvinen, M. (1982). Regulation of the seminiferous epithelium. Endocrine Reviews **3**, 404-417.

Parvinen, M. (1993). Cyclic function of the Sertoli cell. In *The Sertoli Cell* (Eds. L.D. Russell & M.D.Griswold) Cache River Press, Florida. Chapter 14, pp 331-347.

Parvinen, M. & Huhtaniemi, I. (1990). Testosterone micromilieu in staged rat seminiferous tubules. Journal of Steroid Biochemistry **36**, 377-381.

Parvinen, M., Hurme, P. & Niemi, M. (1970). Penetration of exogenous testosterone, pregnenolone, progesterone and cholesterol into the seminiferous tubules of the rat. Endocrinology **87**, 1082-1084.

Parvinen, M., Nikula, H. & Huhtaniemi, I. (1984). Influence of rat seminiferous tubules on Leydig cell testosterone production in vitro. Molecular and Cellular Endocrinology **37**, 331-336.

Parvinen, M., Soder, O., Mali, P., Förysa, B. & Ritzen, E.M. (1991). *In vitro* stimulation of stage specific deoxyribonucleic acid synthesis in rat seminiferous tubule segments by interleukin-1α. Endocrinology **129**, 1614-1620.

Parvinen, M., Vihko, K.K. & Toppari, J. (1986). Cell interactions during the seminiferous epithelial cycle. International Review of Cytology **104**, 115-151.

Persson, H., Ayer-Le Livre, C., Söder, O., Villar, M.J., Metsis, M., Olson, L., Ritzen, M. & Hökfelt, T. (1990). Expression of ß-nerve growth factor receptor mRNA in Sertoli cells downregulated by testosterone. Science **247**, 704-707.

Pierce, J.G. & Parsons, T.F. (1981). Glycoprotein hormones: structure and function. Annual Reviews in Biochemistry **50**, 465-495.

Pineau, C., Sharpe, R.M., Saunders, P.T.K., Gerard, N. & Jégou, B. (1990). Regulation of Sertoli cell inhibin production and of inhibin-α subunit mRNA levels by specific germ cell types. Molecular and Cellular Endocrinology **72**, 13-22.

Pineau, C., Syed, V., Bardin, C.W., Jégou, B. & Cheng, C.Y. (1993). Germ cell-conditioned medium contains multiple factors that modulate the secrtion of testins, clusterin, and transferrin by Sertoli cells. Journal of Andrology **14**, 87-98.

Pineau, C., Velez de la Calle, J.F., Pinon-Lataillade, G. & Jégou, B. (1989). Assessment of testicular function after acute and chronic irradiation: further evidence for an influence of late spermatids on Sertoli cell function in the adult rat. Endocrinology **124**, 2720-2728.

Pinon-Lataillade, G., Velez de la Calle, J.F., Viguier-Martinez, M.C., Garnier, D.H., Folliot, R., Maas, J. & Jégou, B. (1988). Influence of germ cells upon Sertoli cells during continuous low-dose γ-irradiation of adult rats. Molecular and Cellular Endocrinology **58**, 51-63.

Pintar, J.E., Schachter, B.S., Herman, A.B., Durgerian, S. & Krieger, D.T. (1984). Characterization and localization of proopiomelanocortin messenger RNA in the adult rat testis. Science **225**, 632-634.

Plant, T.M., Hess, D.L., Hotchkiss, J. & Knobil, E. (1978). Testosterone and the control of gonadotropin secretion in the male rhesus monkey (*Macaca mulatta*). Endocrinology **103**, 535-541.

Porter, S.B., Ong, D.E., Chytil, F. & Orgebin-Crist, M-C. (1985). Localization of cellular retinol-binding protein and cellular retinoic acid-binding protein in the rat testis and epididymis. Journal of Andrology **6**, 197-212.

Preslock, J.P. (1980). Steroidogenesis in the mammalian testis. Endocrine Reviews **1**, 132-139.

Rajan, N., Blaner, W.S., Soprano, D.R., Suhara, A. & Goodman, D.S. (1990b). Cellular retinol-binding protein messenger RNA levels in normal and retinoid-deficient rats. Journal of Lipid Research **31**, 821-829.

Rajan, N., Sung, W.K. & Goodman, D.S. (1990a). Localization of cellular retinol-binding protein mRNA in rat testis and epididymis and its stage-dependent expression during the cycle of the seminiferous epithelium. Biology of Reproduction **43**, 835-842.

Ratnasooriya, W.D. & Sharpe, R.M. (1989). Evaluation of the effect of selective germ cell depletion on subsequent spermatogenesis and fertility in the rat. International Journal of Andrology **12**, 44-57.

Redding, T.W., Schally, A.V., Arimura, A. & Matsuo, H. (1972). Stimulation of release and synthesis of luteinizing hormone (LH) And follicle stimulating hormone (FSH) in tissue cultures of rat pituitaries in response to natural and synhetic LH and FSH releasinghormone. Endocrinology **90**, 764-770.

Rivier, C., Cajander, S., Vaughan, J., Hsueh, A.J.W. & Vale, W. (1988). Age-dependent changes in physiological action, content, and immunostaining of inhibin in male rats. Endocrinology **123**, 120-126.

Risbridger, G.P., Clements, J., Robertson, D.M., Drummond, A.E., Muir, J., Burger, H.G. & de Kretser, D.M. (1989). Immuno- and bioactive inhibin and inhibin α-subunit expression in rat Leydig cell cultures. Molecular and Cellular Endocrinology **66**, 119-122.

Risbridger, G.P., Kerr, J.B. & de Kretser, D.M. (1981). Evaluation of Leydig cell function and gonadotropin binding in unilateral and bilateral cryptorchidism: evidence for local control of Leydig cell function by the seminiferous tubule. Biology of Reproduction **24**, 534-540.

Roberts, K.P., Awoniy, C.A., Santulli, R. & Zirkin, B.R. (1991). Regulation of Sertoli cell transferrin and sulphated glycoprotein-2 messenger ribonucleic acid levels during restoration of spermatogenesis in the adult hypophysectomised rat. Endocrinology **129**, 3417-3423.

Roberts, K.P. & Griswold, M.D. (1990). Characterization of rat transferrin receptor cDNA: The regulation of transferrin receptor mRNA in testes and in Sertoli cells in culture. Molecular Endocrinology **4**, 531-542.

Roberts, K.P., Santulli, R., Seiden, J. & Zirkin, B.R. (1992). The effect of testosterone withdrawal and subsequent germ cell depletion on transferrin and sulphated glycoprotein-2 messenger ribonucleic acid levels in the adult rat testis. Biology of Reproduction **47**, 92-96.

Roberts, V., Meunier, H., Sawchenko, P.E. & Vale, W. (1989). Differential production and regulation of inhibin subunits in rat testicular cell types. Endocrinology **125**, 2350-2359.

Rodin, D.A., Lalloz, M.R.A. & Clayton, R.N. (1989). Gonadotropin-releasing hormone regulates follicle-stimulating hormone ß-subunit gene expression in the male rat. Endocrinology **125**, 1282-1289.

Rommerts, F.F.G. & Cooke, B.A. (1988). The mechanisms of action of luteinising hormone II. Transducing systems and biological effects. In *Hormones and their Actions, Part I.* (Eds. B.A. Cooke, R.J.B. King & H.J. van der Molen). Elsevier Science Publishers.

Rommerts, F.F.G., de Jong, F.H., Brinkman, A.O. & van der Molen, H.J. (1982). Development and cellular localisation of rat testicular aromatase activity. Journal of Reproduction and Fertility **65**, 281-288.

Rommerts, F.F.G., Druger-Sewnarian, B.Ch., van Woerkom-Blik, A., Grootegoed, J.A. & van der Molen, H.J. (1978). Secretion of proteins by Sertoli cell enriched cultures: effects of follicle stimulating hormone, dibutyryl cAMP and testosterone and correlation with secretion of oestradiol and androgen binding protein. Molecular and Cellular Endocrinology **10**, 39-55.

Romrell,L.J. & Ross, M.H. (1979). Characterization of Sertoli cell-germ cell junctional specializations in dissociated testicular cells. Anatomical Record **193**, 23-42.

Russell, L.D. (1977). Observations on rat Sertoli ectoplasmic ('junctional') specializations in their association with germ cells of the rat testis. Tissue Cell. **9**, 475-498.

Russell, L.D. (1979). Spermatid-Sertoli tubulobulbar complexes as devices for elimination of cytoplasm from the head region of late spermatids of the rat. Anatomical Record **194**, 233-246.

Russell, L.D. (1980). Sertoli-germ cell interrelations: a review. Gamete Research **3**, 179-202.

Russell, L.D. (1991). The perils of sperm release-'let my children go'. International Journal of Andrology **14**, 307-311.

Russell, L.D. (1993a). Form, dimensions, and cytology of mammalian Sertoli cells. In *The Sertoli Cell* (Eds. L.D. Russell and M.D.Griswold) Cache River Press, Florida. Chapter 1, pp 1-37.

Russell, L.D. (1993b). Role in spermiation. In *The Sertoli Cell* (Eds. L.D. Russell and M.D.Griswold) Cache River Press, Florida. Chapter 12, pp. 269-303.

Russell, L.D. (1993c). Morphological and functional evidence for Sertoli-germ cell relationships. In *The Sertoli Cell* (Eds. L.D. Russell and M.D.Griswold) Cache River Press, Florida. Chapter 16, pp.365-390.

Russell, L.D., Alger, L.E. & Nequin, L.G. (1987). Hormonal control of pubertal spermatogenesis. Endocrinology **120**, 1615-1632.

Russell, L.D., Bartke, A. & Goh, J.C. (1989). Postnatal development of the Sertoli cell barrier, tubular lumen, and cytoskeleton of Sertoli and myoid cells in the rat, and their relationship to tubular fluid secretion. The American Journal of Anatomy **184**, 179-189.

Russell, L.D. & Malone, J.P. (1980). A study of Sertoli-spermatid tubulobulbar complexes in selected mammals. Tissue Cell **12**, 263-285.

Russell, L.D. & Peterson, R.N. (1984). Determination of the elongate spermatid-Sertoli cell ratio in various mammals. Journal of Reproduction and Fertility **70**, 635-641.

Russell, L.D., Saxena, N.K. & Tarner, T.T. (1989). Cytoskeletal involvement in spermiation and sperm transport. Tissue Cell **21**, 361-379.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**, 487-491.

Sairam, M.R. (1989). Role of carbohydrates in glycoprotein hormone signal transduction. FASEB Journal **3**, 1915-1926.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd Ed.Cold Spring Harbour Press, Cold Spring Harbour, New York.

Sanborn, B.M., Caston, L.A., Chang, C., Liao, S., Speller, R., Porter, L.D. & Ku, K.U. (1991). Regulation of androgen receptor mRNA in rat Sertoli and peritubular cells. Biology of Reproduction **45**, 634-641.

Sanborn, B.M., Steinberger, A., Meistreich, M.L. & Steinberger, E. (1975). Androgen binding sites in testis cell fractions as measured by a nuclear exchange assay. Journal of Steroid Biochemistry **6**, 1459-1465.

Sanchéz-Yagüe, J., Rodriguez, M.C., Segaloff, D.L. & Ascoli, M. (1992). Truncation of the cytoplasmic tail of the lutropin choriogonadotropin receptor prevents agonist induced uncoupling. Journal of Biological Chemistry **267**, 7217-7220.

Sar, M., Lubahn, D.B., French, F.S. & Wilson, E.M. (1990). Immunohistochemical localization of the androgen receptor in rat and human tissues. Endocrinology **127**, 3180-3186.

Saunders, P.T.K., Millar, M.R., Maguire, S.M. & Sharpe, R.M. (1992). Stage-Specific Expression of Rat Transition Protein 2 mRNA and Possible Localisation to the Chromatoid Body of Step 7 Spermatids by In Situ Hybridisation Using a Non-Radioactive Riboprobe. Molecular Reproduction and Development **33**, 385-391.

Saunders, P.T.K., Millar, M.R., West, A.P. & Sharpe, R.M. (1993). Mitochondrial cytochrome c oxidase II messenger ribonucleic acid is expressed in pachytene spermatocytes at high levels and in a stage-dependent manner during spermatogenesis in the rat. Biology of Reproduction **48**, 57-67.

Schulze, W. & Rehder. (1984). Organisation and morphogenesis of the human seminiferous epithelium. Cell and Tissue Research **237**, 395-407.

Scoot, R.S. & Burger, H.G. (1981). Mechanism of action of inhibin. Biology of Reproduction **24**, 541-550.

Setchell, B.P. (1969). Do Sertoli cells secrete fluid into the seminiferous tubules? Journal of Reproduction and Fertility **19**, 391-392.

Setchell, B.P. (1990). Local control of testicular fluids. Reproduction, Fertility and Development **2**, 291-309.

Shaha, C., Morris, P.L., Chen, C-L.C., Vale, W. & Bardin, C.W. (1989). Immunostainable inhibin subunits are in multiple types of testicular cells. Endocrinology **125**, 1941-1950.

Shan, B., Vazquez, E. & Lewis, J.A. (1990). Interferon selectively inhibits the expression of mitochondrial genes: a novel pathway for interferon-mediated responses. The EMBO Journal **9**, 4307-4314.

Sharpe, R.M. (1988). Bidirectional secretion by the Sertoli cell. International Journal of Andrology **11**, 87-91.

Sharpe, R.M. (1989). Possible role of elongate spermatids in control of stage-dependent changes in the diameter of the lumen of the rat seminiferous tubule Journal of Andrology **10**, 304-310.

Sharpe, R.M. (1992). Are environmental chemicals a threat to male fertility? Chemistry and Industry ?? **3rd** February Issue, 88-92.

Sharpe, R.M. (1993) Experimental evidence for Sertoli-germ cell and Sertoli-Leydig cell interactions. In *The Sertoli Cell* (Eds. L.D. Russell and M.D.Griswold) Cache River Press, Florida. Chapter 17, pp 391-418.

Sharpe, R.M. (1994). Regulation of spermatogenesis. In *The Physiology of Reproduction* (Eds. E.Nobil & J.D.Neill). Raven Press, New York. In Press.

Sharpe, R.M., Donachie, K & Cooper, I. (1988). Re-evaluation of the intratesticular level of testosterone required for quantitative maintenance of spermatogenesis in the rat. Journal of Endocrinology **117**, 19-26.

Sharpe, R.M., Doogan, D.G. & Cooper, I. (1983). Direct effects of a luteinising hormone-releasing hormone agonist on intratesticular levels of testosterone and interstitial fluid formation in intact male rats. Endocrinology **113**, 1306-1313.

Sharpe, R.M., Fraser, H.M., Cooper, I. & Rommerts, F.F.G. (1981). Sertoli-Leydig cell communication via an LHRH-like factor. Nature **290**, 785-787.

Sharpe, R.M., McKinnell, C., Millar, M,. Maguire, S. & Saunders, P.T.K. (1993). Leydig cell-Sertoli cell-germ cell interactions. In *Understanding Male Infertility: Basic and Clinical Approaches..* (Eds. R. Whitcomb and B. Zirkin) Raven Press. Serono Symposia Vol. 98, pp 143-153.

Sharpe, R.M., Millar, M & McKinnell, C. (1993). Relative roles of testosterone and the germ cell complemt in determining stage dependent chnges in protein secretion by isolated rat semiiferous tubules. International Journal of Andrology **16**, 71-81.

Sharpe, R.M. & Skakkebaek, N.E. (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? The Lancet **341**, 1392-1395.

Sherman, D.R., Lloyd, R.S. & Chytil, F. (1987). Rat cellular retinol-binding protein: cDNA sequence and rapid retinol-depemdent accumulation of mRNA. Proceedings of the National Academy of Sciences, USA **84**, 3209-3213.

Shubhada, S., Glinz, M. & Lamb, D.J. (1993). Sertoli cell secreted growth factor. Cellular origin, paracrine and endocrine regulation of secretion. Journal of Andrology **14**, 99-109.

Sive, H.L. & St. John, T. (1988). A simple subtractive hybridisation technique employine photoactivatable biotin and phenol extraction. Nucleic Acid Research **16**, 10937.

Skinner, M.K., Fetterolf, P.M. & Anthony, C.T. (1988). Purification of a paracrine factor, P-Mod-S, produced by testicular peritubular cells that modulates Sertoli cell function. The Journal of Biological Chemistry **263**, 2884-2890.

Skinner, M.K. & Fritz, I.B. (1985). Androgen stimulation of Sertoli cell function is enhanced by peritubular cells. Molecular and Cellular Endocrinology **40**, 115-122.

Skinner, M.K. & Griswold, M.D. (1980). Sertoli cells synthesize and secrete transferrin-like protein. Journal of Biological Chemistry **255**, 9523-9525.

Skinner, M.K. & Griswold, M.D. (1983). Sertoli cells synthesize and secrete ceruloplasmin-like protein. Biology of Reproduction **28**, 1225-1229.

Skinner, M.K., McLachlan, R.I. & Bremner, W.J. (1989c). Stimulation of Sertoli cell inhibin secretion by the testicular paracrine factor PModS. Molecular and Cellular Endocrinology **66**, 239-249.

Skinner, M.K. & Moses, H.L. (1989). Transforming growth factor ß gene expression and action in the seminiferous tubule: peritubular cell-Sertoli cell interaction. Molecular Endocrinology **3**, 625-634.

Skinner, M.K., Schlitz, S.M. & Anthony, C.T. (1989a) Regulation of Sertoli cell differentiated function: testicular transferrin and androgen binding protein expression. Endocrinology **124**, 3015-3024.

Skinner, M.K., Takacs, K. & Coffey, R.J. (1989b). Transforming growth factor-α gene expression and action in the seminiferous tubule: peritubular cell-Sertoli cell interactions. Endocrinology **124**, 845-854.

Skinner, M.K., Tung, P.S. & Fritz, I.B. (1985). Cooperativity between Sertoli cells and testicular peritubular cells in the production and deposition of extracellular matrix components. The Journal of Cell Biology **100**, 1941-1947.

Smith, P.E. (1930). Hypophysectomy and a replacement therapy in the rat. The American Journal of Anatomy **45**, 205-273.

Sordoillet, C. Chauvin, M.A., Revol, A., Morera, A.M. & Benahmed, M. (1988). Fibroblast growth factor is a regulator of testosterone secretion in cultured immature Leydig cells. Molecular and Cellular Endocrinology **58**, 283-286.

Sprengel, R., Braun, T., Nikolics, K., Segaloff, D.L. & Seeburg, P.H. (1990). The testicular receptor for follicle stimulating hormone: structure and functional expression of cloned cDNA. Molecular Endocrinology **4**, 525-530.

Srinath, B.R., Wickings, E.J., Witting, C. & Nieschlag, E. (1983). Active immunization with follicle-stimulating hormone for fertility control: a $4^1/2$-year study in male rhesus monkeys. Fertility and Sterility **40**, 110-117.

Stallard, B.J., Collard, M.W. & Griswold, M.D. (1991). A transferrinlike (hemiferrin) mRNA is expressed in the germ cells of rat testis. Molecular and Cellular Biology **11**, 1448-1453.

Stallard, B.J. & Griswold, M.D. (1990). Germ cell regulation of Sertoli cell transferrin mRNA levels. Molecular Endocrinology **4**, 393-401.

Steinberger, A., Hintz, M. & Heindel, J.J. (1978). Changes in cyclic AMP responses to FSH in isolated rat Sertoli cells during sexual maturation. Biology of Reproduction **19**, 566-572.

Strosberg, A.D. (1991). Structure/fuction relationship of proteins belonging to the family of receptors of coupled to GTP-binding proteins. European Journal of Biochemistry **196**, 1-10.

Suk Han, I., Sylvester, S.R., Hee Kim, K., Schelling, M.E., Venkateswaran, S., Blanckaert, V.D., McGuinness, M.P. & Griswold, M.D. (1993). Basic fibroblast growth factor is a testicular germ cell product which may regulate Sertoli cell function. Molecular Endocrinology **7**, 889-897.

Syed, V., Söder, O., Arver, S., Lindh, M., Khan, S. & Ritzén, M. (1988). Ontogeny and cellular origin of an interleukin-1 like factor in the reproductive tract of the male rat. International Journal of Andrology **11**, 437-447.

Sylvester, S.R. & Griswold, M.D. (1984). Localization of transferrin and transferrin receptors in rat testes. Biology of Reproduction **31**, 195-203.

Sylvester, S.R., Morales, C.R., Oko, R. & Griswold, M.D. (1989). Sulphated glycoprotein-1 (saposin precursor) in the reproductive tract of the male rat. Biology of Reproduction **41**, 941-948.

Sylvester, S.R., Morales, C.R., Oko, R. & Griswold, M.D. (1991). Localization of sulphated glycoprotein-2 (clusterin) on spermatozoa and in the reproductive tract of the male rat. Biology of Reproduction **45**, 195-207.

Sylvester, S.R., Skinner, M.K. & Griswold, M.D. (1984). A sulphated glycoprotein synthesised by Sertoli cells and by epididymal cells is a component of the sperm membrane. Biology of Reproduction **31**, 1087-1101.

Tavéra, C., Prévot, D., Girolami, J.P., Leung-Tack, J. & Collé, A. (1990). Tissue and biological fluid distribution of cysteine proteinases inhibitor: rat cystatin C. Biological Chemistry Hoppe-Seyler Supplement **371**, 187-192.

Teerds, K.J., Rommerts, F.F.G. & Dorrington, J.H. (1990). Immnohistochemical detection of trasforming growth factor-α in Leydig cells during the development of the rat testis. Molecular and Cellular Endocrinology **69**, R1-R6.

Tindall, D.J. & Means, A.R. (1976). Concerning the hormonal regulation of androgen binding protein in rat testis. Endocrinology **99**, 809-818.

Tindall, D.J., Vitale, R. & Means, A.R. (1975). Androgen binding protein as a biochemical marker of formation of the blood-testis barrier. Endocrinology **97**, 636-648.

Toebosch, A.M.W., Robertson, D.M., Klaij, I.A., de Jong, F.H. & Grootegoed, J.A. (1989). Effects of FSH and testosterone on highly purified rat Sertoli cells: inhibin α-subunit mRNA expression and inhibin secretion are enhanced by FSH but not by testosterone. Journal of Endocrinology **122**, 757-762.

Tres, L.L. & Kierszenbaum, A.L. (1983). Viability of rat spermatogenic cells *in vitro* is facilitated by their coculture with Sertoli cells in serum-free hormone-supplemented medium. Proceedings of the National Academy of Sciences USA **80**, 3377-3381.

Tsong, S.D., Phillips, D., Halmi, N., Liotta, A.S., Margioris, A., Bardin, C.W. & Krieger, D.T. (1982). ACTH and ß-endorphin related peptides are present in multiple sites in the reproductive tract of the male rat. Endocrinology **110**, 2204-2206.

Tsuruta, J.K., O'Brien, D.A. & Griswold, M.D. (1993). Sertoli cell and germ cell cystatin C: stage-dependent expression of two distinct messenger ribonucleic acid transcripts in rat testis. Biology of Reproduction **49**, 1045-1054.

Tsutsumi, M., Zhou, W., Millar, R.P., Mellon, P.L., Roberts, J.L., Flanagan, C.A., Dong, K., Gillo, B. & Sealfon, S.C. (1992). Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Molecular Endocrinology **6**, 1163-1169.

Ueno, H. & Mori, H. (1990). Morphometrical analysis of Sertoli cell ultrasttructure during the seminiferous epithelial cycle in rats. Biology of Reproduction **43**, 769-776.

Uzan, G., Frain, M., Park, I., Besmond, C., Maessen, G., Trepat, J.S., Zakin, M.M. & Kahn, A. (1984). Molecular cloning and sequence analysis of cDNA for human transferrin. Biochemical and Biophysical Research Communications **119**, 273-281.

van Alphan, M.M.A., van de Kant, H.J.G. & de Rooij, D.G. (1988). Follicle-stimulating hormone stimulates spermatogenesis in the adult monkey. Endocrinology **123**, 1449-1455.

van Dissel-Emiliani, F., Grootenhuis, A., de Jong, F. & de Rooij, D. (1989). Inhibin reduces spermatogonial numbers in testes of adult mice and Chinese hamsters. Endocrinology **125**, 1899-1903.

van Noort, M., Rommerts, F.F.G., van Amerongen, A. & Wirtz, K.W.A. (1988). On the role of SCP_2 in hormonal regulation of steroid production in Leydig cells. In *The Molecular Endocrinology of the Testis*. (Eds. B.A. Cooke & R.M. Sharpe). Serono Symposia Publications from Raven Press Volume 50, pp103-109.

Vaughan, L., Carmel, P.W., Dyrenfurth, I., Frantz, A.G., Antunes, J.L. & Ferin, M. (1980). Section of the pituitary stalk in the rhesus monkey. 1. Endocrine studies. Neuroendocrinology **30**, 70-75.

Verhoeven, G. & Cailleau, J. (1988). Testicular peritubular cells secrete a protein under androgen control that inhibits induction of aromatase activity in Sertoli cells. Endocrinology **123**, 2100-2110.

Vitale, R., Fawcett, D.W. & Dym, M. (1973). The normal development of the blood testis barrier and the effects of clomiphene and estrogen treatment. Anatomical Record **176**, 333-344.

Waites, G.M.H. & Gladwell, R.T. (1982). Phsiological significance of fluid secretion in the testis and blood-testis barrier. Physiological Reviews **62**, 624-671.

Waites, G.M.H., Speight, A.C. & Jenkins, N. (1985). The functional maturation of the Sertoli cell and Leydig cell in the mammalin testis. Journal of Reproduction and Fertility **75**, 317-326.

Wang, H., Segaloff, D.L. & Ascoli, M. (1991). Lutropin/ choriogonadotropin down-regulates its receptor by both receptor mediated endocytosis and a cAMP-dependent reduction in receptor mRNA. Journal of Biological Chemistry **266**, 780-785.

Ward, W.S. & Coffey, D.S. (1991). DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. Biology of Reproduction **44**, 569-574.

Warren, D.W., Huhtaniemi, I.H., Tapanainen, J., Dufau, M.L. & Catt, K.J. (1984). Ontogeny of gonadotropin receptors in the fetal and neonatal rat testis. Endocrinology **114**, 470-475.

Warren, D.W., Pasupuleti, V., Lu, Y., Platler, B.W. & Horton, R. (1990). Tumor necrosis factor and interleukin-1 stimulate testosterone secretion in adult male rat Leydig cells in vitro. Journal of Andrology **11**, 353-360.

Wierman, M.E., Gharib, S.D., LaRovere, J.M., Badger, J.M. & Chin, W.W. (1989). Selective failure of androgens to regulate follicle-stimulating hormone ß mRNA levels in the male rat. Molecular Endocrinology **2**, 492-498.

Williams, J. & Foster, P.M.D. (1988). The production of lactate and pyruvate as sensitive indices of altered rat Sertoli cell function in vitro following addition of various testicular toxicants. Toxicology and Applied Pharmacology **94**, 160-170.

Woodruff, T.K., Borree, J., Attie, K.M., Cox, E.T., Rice, G.C. & Mather, J.P. (1992). Stage-specific binding of inhibin and activin to subpopulations of rat germ cells. Endocrinology **130**, 871-881.

Woodruff, T.K., Meunier, H., Jones, P.B.C., Hsueh, A.J.W. & Mayo, K.E. (1987). Rat inhibin: molecular cloning of α- and ß-subunit complementary deoxyribonucleic acids and expression in the ovary. Molecular Endocrinology **1**, 561-568.

Wright, W.W. (1988). Germ cell-Sertoli cell interactions: analysis of the biosynthesis and secretion of cyclic protein-2. Developmental Biology **130**, 45-56.

Wright, W.W. & Frankel, A.I. (1980). An androgen receptor in the nuclei of late spermatids in testes of male rats. Endocrinology **107**, 314-317.

Wright, W.W. & Lazarraga, M.L. (1986). Isolation of cyclic protein-2 from rat seminiferous tubule fluid and Sertoli cell culture medium. Biology of Reproduction **35**, 761-772.

Wright, W.W., Parvinen, M., Musto, N.A., Gunsalus, G.L., Phillips, D.M., Mather, J.P. & Bardin, C.W.(1983). Identification of stage specific proteins synthesised by rat seminiferous tubules. Biology of Reproduction **29**, 257-270.

Wright, W.W., Zabludoff, S.D., Erickson-Lawrence, M. & Karzai, A.W. (1989). Germ cell-Sertoli cell interactions. Studies of cyclic protein-2 in the seminiferous epithelium. Annals of the New York Academy of Sciences **564**, 173-185.

Ying, S-Y. (1988). Inhibins, Activins and Follistatins: Gonadal proteins modulaing the secretion of follicle-stimulating hormone. Endocrine Reviews **9**, 267-293.

Yoon, D.J., Reggiardo, D. & David, R. (1990). Available FSH receptors in adult rat testis *in vivo*. Journal of Endocrinology **125**, 293-299.

Zabludoff, S.D., Erickson-Lawrence, M. & Wright, W.W. (1990a). Sertoli cells, proximal convoluted tubules in the kidney, and neurons in the brain contain cyclic protein-2. Biology of Reproduction **43**, 15-24.

Zabludoff, S.D., Karzai, A.W.. & Wright, W.W. (1990b). Germ cell-Sertoli cell interactions: the effect of testicular maturation on the synthesis of cyclic protein-2 by rat Sertoli cells. Biology of Reproduction **43**, 25-33.

Zakeri, Z., Curto, M., Hoover, D., Wightman, K., Engelhardt, J., Kierszenbaum, A.L., Gleeson, T. & Tenniswood, M. (1992). Developmental expression of the S35-S45/SGP-2/TRPM-2 gene in rat testis and epididymis. Molecular Reproduction and Development **33**, 373-384.

Ziparo, E., Geremia, R., Russo, M.A. & Stefanini, M. (1980). Surface interaction *in vitro* between Sertoli cells and germ cells of different stages of spermatogenesis. American Journal of Anatomy **159**, 385-388.

Molecular and Cellular Endocrinology, 94 (1993) 79–88

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Stage-dependent expression of mRNA for cyclic protein 2 during spermatogenesis is modulated by elongate spermatids

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Summary

Cyclic protein 2 (CP-2) is a product of the Sertoli cell which is secreted in a cyclical manner according to the stage of the spermatogenic cycle. This study has assessed the influence of the germ cell complement on expression of CP-2 mRNA. Adult rats were treated with 650 mg/kg methoxyacetic acid (MAA) to induce the specific depletion of > 80% of pachytene and later spermatocytes from most tubules, and expression of CP-2 mRNA was then assessed at various times after treatment when particular germ cell types were depleted selectively. CP-2 mRNA was specifically localised to the Sertoli cells of the seminiferous tubules by non-radioactive in situ hybridisation using a digoxigenin-labelled riboprobe. A stage specific variation in CP-2 mRNA levels was observed, with the mRNA being most abundant at stages IV–VII of the spermatogenic cycle. Northern analysis revealed that treatment with MAA led to an apparent increase in the amount of the major 1.7 kb CP-2 transcript when either pachytene spermatocytes or round spermatids were depleted. In contrast, the level of CP-2 mRNA was decreased by more than half at 21 days after MAA treatment. This decrease was confirmed by in situ hybridisation at 21 days after MAA treatment, when CP-2 mRNA expression was found to be decreased or absent from tubules at stages at which CP-2 mRNA is normally expressed (stages IV–VII) when elongate spermatids were depleted selectively from these tubules. These observations lead us to hypothesise that elongate spermatids positively modulate CP-2 mRNA expression in the Sertoli cell.

Introduction

In the rat the spermatogenic cycle has been divided into fourteen stages (Leblond and Clermont, 1952). At each stage there is a fixed complement of 4 or 5 types of germ cells which are in intimate contact with the Sertoli cells. The multiplication, development and differentiation of the germ cells is thought to be dependent on the supply of various factors by the Sertoli cell. The function of the Sertoli cell changes dramatically according to the stage of the spermatogenic cycle, and there are many Sertoli cell products which are produced and/or secreted in a cyclical pattern (Parvinen, 1992). There is growing evidence that this cyclicity of function of the Sertoli cell is a consequence of the changing germ cell complement with which the Sertoli cell is associated at the different stages (Parvinen, 1992; Sharpe, 1992). Studies both in vivo and in vitro have shown that the secretion of Sertoli cell products, such as transferrin, androgen-binding protein (ABP), oestradiol and inhibin can be regulated by particular germ cell types (Onoda and Djakiew, 1990; Le Magueresse and Jégou, 1988a, b; Pineau et al., 1990; Allenby et al., 1991). This regulation may occur at several levels including that of transcription.

Cyclic protein 2 (CP-2) is a major product of the Sertoli cell and is known to be secreted maximally at stages VI and VII of the spermatogenic cycle (Wright et al., 1983). A partial cDNA for CP-2 mRNA has been cloned recently and was shown to code for the proenzyme form of cathepsin L, a cysteine protease (Erickson-Lawrence et al., 1991). These authors proposed that CP-2/cathepsin L may act at stages VI/VII of the spermatogenic cycle to degrade adhesion molecules which bind elongate spermatids to the Sertoli cell thus allowing movement of these spermatids from the base to the lumen of the seminiferous tubule

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in preparation for their release. A cyclical pattern of secretion of a product, such as CP-2, by the Sertoli cell is a strong indication that the production/secretion of this factor is germ cell-modulated. The aim of the present study was to assess whether the levels of mRNA for CP-2 in the Sertoli cell were affected by selective germ cell depletion from seminiferous tubules. This was achieved using the testicular toxicant methoxyacetic acid (MAA) which selectively depletes seminiferous tubules of pachytene and later spermatocytes and then assessing the levels of mRNA for CP-2 at selected time points after treatment when particular germ cell types (pachytene spermatocytes, round spermatids or elongate spermatids) were missing (see Bartlett et al. 1988; Allenby et al., 1991).

Materials and methods

Reagents

All molecular biology grade chemicals were from Sigma Chemical Co., Poole, Dorset, UK or IBI Ltd., Cambridge, UK. Enzymes and buffers were from Promega Ltd., Southampton, UK or Boehringer Mannheim UK, Lewes, Sussex. Radiolabelled nucleotides were from Amersham, Slough, Berks.

Animals and treatments

Adult Wistar rats (aged 70–85 days) were administered methoxyacetic acid (MAA, Aldrich Chemical Co. Ltd.) as a single oral dose of 650 mg/kg. Administration of MAA at this dose level results in the selective depletion of 80–100% of pachytene and later spermatocytes at all stages of the spermatogenic cycle except early to mid stage VII (Bartlett et al., 1988). Spermatogenesis proceeds with normal kinetics such that at selected time points after MAA treatment round and then elongate spermatids are selectively absent from the testis because of maturation depletion (Bartlett et al., 1988; Allenby et al., 1991). In this study MAA-treated animals were killed at 3, 7, 14, 21, 28 and 42 days after treatment. At each time point animals were either perfusion fixed with Bouins fluid via the dorsal aorta and the tissue processed for in situ hybridisation (Millar et al., 1993) or the testes were removed for RNA extraction and Northern analysis (see below).

Preparation of a partial cDNA clone for CP-2

A partial cDNA clone for CP-2 was amplified by polymerase chain reaction (PCR; Saiki et al., 1988) from a pool of rat testis cDNAs prepared by random priming of mRNAs. Oligonucleotide primers were selected to amplify from base 6 to base 499 of the published sequence of CP-2 (Erickson-Lawrence et al., 1991). Primers were synthesised on a model 381 oligonucleotide synthesiser (Applied Biosystems, Warrington) using phosphoramidite chemistry, eluted into ammonia, recovered by precipitation, dissolved in water and used without further purification. The sequences were as follows, 5′ primer (sense strand) 5′-AGG CAG ATA GTG AAT GGC TAT CG-3′ (bases 6–28) and 3′ primer (antisense strand) 5′-T GGC TTG CAT CCA TGG CAA CAG-3′ (bases 477–499), and had the sites for *Eco*RI and *Hin*dIII, respectively added to their 5′ ends. The primers were used at a concentration of 0.5 μM in a PCR reaction containing 100 μM each dNTP (Pharmacia), Taq polymerase buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100) and 2.5 U Taq polymerase (Promega). Thirty-five cycles of amplification were performed with an annealing temperature of 61°C and a 1 min extension at 72°C. A single 493 bp cDNA was amplified and purified using the Qiagen PCR purification kit (Diagen). To confirm that it was identical to the published sequence the purified cDNA was sequenced by chain termination using Sequenase (USB) and methods described previously (Saunders et al., 1992a). The purified partial CP-2 cDNA was digested with *Eco*RI and *Hin*dIII, extracted with phenol/chloroform, precipitated and ligated into Bluescript SK^- (Stratagene) which had been linearised with the same enzymes. Plasmids were transformed into *E. coli* XL1-blue, amplified in liquid culture, purified with a Promega miniprep kit and used to prepare labelled riboprobes (see below).

A cDNA clone for transition protein 2 (TP-2), the mRNA of which is expressed specifically in step 7 to step 13 spermatids, was prepared as described in Saunders et al. (1992b).

RNA Extraction and Northern analysis

RNA was extracted from the testes of control and MAA-treated animals (2 per treatment day) according to the method of Chomczynski and Sacchi (1987). Total RNA (15 or 20 μg per lane) was separated using standard procedures on 1.5% denaturing agarose gels containing formaldehyde (Davis et al., 1986). RNA was transferred onto Hybond-N membranes (Amersham) by capillary blotting using 20 × SSC (1 × SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate at pH 7.0) and fixed to the membrane by ultraviolet light.

To provide material for radiolabelling the partial cDNAs for CP-2 and TP-2 were amplified from the plasmids described above by PCR using SK and KS primers corresponding to regions of DNA flanking the multiple cloning site in Bluescript. DNA was labelled with ^{32}P-dCTP by random priming (Feinberg and Vogelstein, 1983). Membranes were prehybridised for at least 2 h in 0.2 M phosphate buffer pH 7.2 containing 1% BSA, 7% SDS and 15% deionised formamide at 65°C. Labelled cDNAs were added at a final concentration of $0.5–1 \times 10^6$ cpm/ml and hybridisation continued for 16–24 h. Membranes were washed at 65°C with 40 mM phosphate buffer containing 1% SDS (2×30 min) and exposed to X-ray film (X-AR 5 or X-OmatS, Kodak) for 24–48 h. To check for even transfer of RNA, membranes were stripped with $0.1 \times$ SSC at 100°C and reprobed using an antisense oligonucleotide for 18S ribosomal RNA (Chang et al., 1984; Saunders et al., 1992b) labelled at the 3′ end with ^{32}P-gamma-ATP according to standard methods (Sambrook et al., 1989).

In situ hybridisation

DNA templates for riboprobe synthesis were prepared by digestion of Bluescript containing the 493 bp CP-2 cDNA with either *Bam*HI or *Hin*dIII restriction

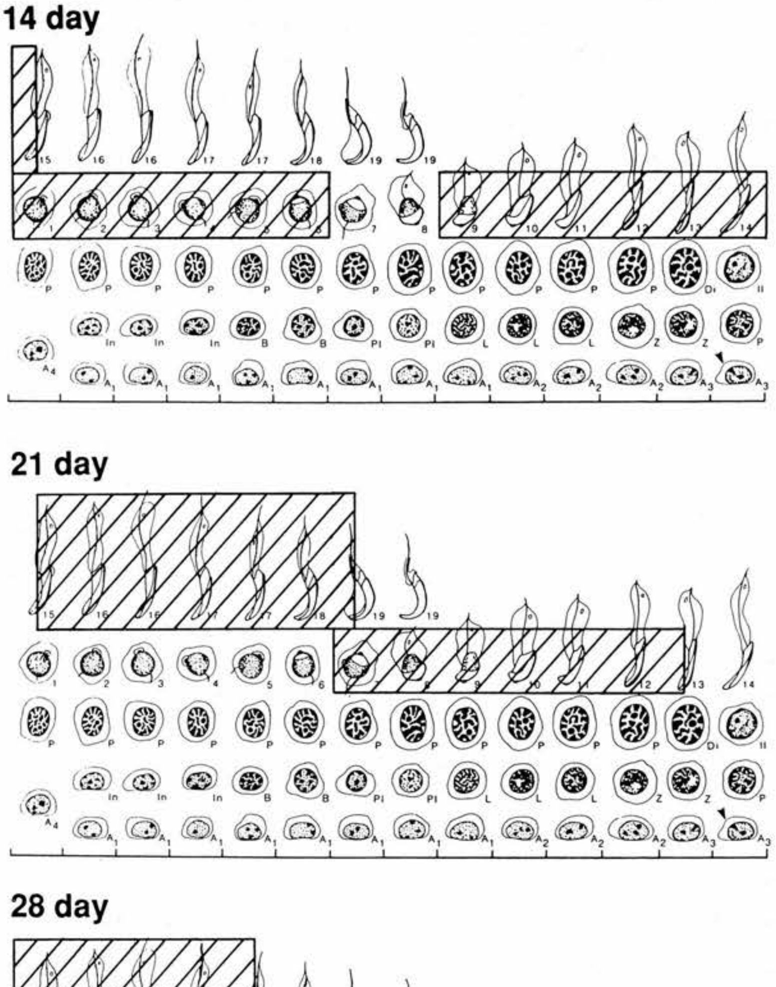

Fig. 1. Pattern of depletion of germ cell types observed at 14, 21 and 28 days after administration of a single oral dose of 650 mg/kg methoxyacetic acid (MAA). The stages (I–XIV) of the spermatogenic cycle and their duration in hours are shown at the bottom (based on Leblond and Clermont, 1952). MAA treatment initially causes a loss of 80–100% pachytene and later spermatocytes at all stages of the spermatogenic cycle with the exception of early to mid stage VII. As spermatogenesis progresses this initial lesion results in a selective loss of round and then elongate spermatids.

enzymes to linearise the plasmid. Antisense and sense riboprobes labelled with ^{35}S-UTP or digoxigenin-UTP were prepared by in vitro transcription using T3 or T7 RNA polymerase, respectively for 1–2 h at 37°C according to the methods detailed in Millar et al. (1993).

Localisation of the mRNA for CP-2 by in situ hybridisation utilised sections of testis from both control and MAA-treated animals embedded in paraffin and sectioned at 5 μm. Pretreatment of sections was as described in Millar et al. (1993). Briefly, sections were cleared in histoclear (National Diagnostics, Manville, New Jersey, USA), dehydrated and placed in 0.2 N HCl for 20 min at room temperature. The sections were incubated in 2 μg/ml proteinase K at 37°C for 20 or 40 min (radiolabelled and non-radioactive probes respectively) followed by 0.2% glycine at 4°C for 10 min, and acetylated for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine pH 8.0. Thereafter sections were prehybridised in buffer containing 4 × STE (1 × STE contains 150 mM NaCl, 2.5 mM Tris and 0.25 mM EDTA), 1 × Denhardt's solution (Sambrook et al., 1989), 125 μg/ml salmon sperm DNA, 125 μl/ml yeast tRNA and either 37% (non-radioactive) or 50% (radiolabelled) formamide at 50°C for 2–4 h. Hybridisation was continued overnight at 50°C after addition of probe to a concentration of either 1×10^6 cpm in 40 μl buffer/slide for ^{35}S-UTP-labelled riboprobes or 200 ng/ml buffer for Dig-labelled riboprobes; the hybridisation buffer was prehybridisation buffer plus 10% dextran sulphate.

Post hybridisation washes were as detailed in Millar et al. (1993). Sections hybridised with radiolabelled CP-2 cRNA were washed briefly in 4 × SSC (10 min) to remove the coverslip and then treated with RNase A at a concentration of 20 μg/ml for 30 min at 37°C. Subsequent washes of RNase buffer alone at 37°C for 30 min, 2 × SSC at room temperature and 45°C for 30 min each, and a final wash of 0.5 × SSC at room temperature were carried out to remove non-hybridised probe. Slides were dipped in NTB-2 emulsion (Kodak), stored in the dark at 4°C and developed after 7 days. After hybridisation with a digoxigenin-labelled riboprobe sections were washed briefly with 4 × SSC (10 min) and treated with 20 μg/ml RNase A as above. Excess probe was removed by 2 × 5 min washes with 2 × SSC at room temperature and 15 min in 0.1 × SSC/30% formamide at 40°C. Visualisation of hybridised probe was achieved with an anti-digoxigenin-alkaline phosphatase (AP)-antibody conjugate (Boehringer Mannheim, Lewes, Sussex). The bound antibody was visualised by an enzyme catalysed (AP) colour reaction with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, X-phosphate) and nitroblue tetrazolium chloride (NBT; Millar et al., 1993). The reaction was

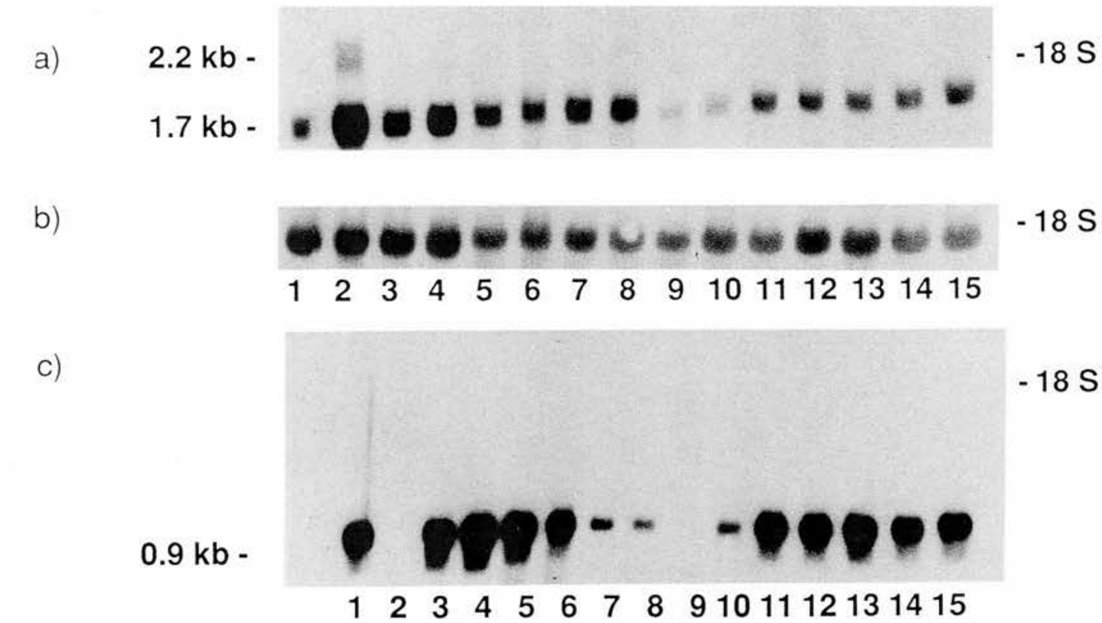

Fig. 2. Northern blot analysis of CP-2 mRNA expression in samples from a control rat testis (lanes 1 and 15) and kidney (lane 2) and testes from each of two animals administered a single oral dose of methoxyacetic acid 3, 7, 14, 21, 28 or 42 days previously (lanes 3–4, 5–6, 7–8, 9–10, 11–12 and 13–14, respectively). All lanes were loaded with 15 μg total RNA. (A) The membrane was hybridised with ^{32}P-labelled CP-2 cDNA and exposed to X-Omat AR film for 48 h. (B) The membrane was stripped and reprobed with an oligonucleotide probe for the 18S ribosomal RNA to check that all lanes contained equivalent amounts of RNA (C). The membrane was stripped again and reprobed with a ^{32}P-labelled TP-2 cDNA to confirm loss of condensing spermatids due to MAA treatment.

allowed to continue for up to 24 h until the colour had developed.

Results

Testicular morphology

Examination of cross-sections of testes from rats at all time points after MAA treatment confirmed the expected specific depletion of the different germ cell types. MAA treatment resulted initially in the depletion of pachytene and later spermatocytes at all stages of the spermatogenic cycle except early to mid stage VII. The pattern and degree of depletion was comparable to that reported previously (Bartlett et al., 1988) as was the resistance of pachytene spermatocytes at early- to mid-stage VII to the adverse effects of MAA. These 'resistant' cells proceeded through spermatogenesis with normal kinetics such that at each time point after MAA-treatment, the stages of the spermatogenic cycle containing these cells exhibited a full complement of germ cells. The cell types depleted at selected time points after treatment are summarised in Fig. 1.

Northern analysis

Northern blot analysis of testicular RNA with a partial cDNA clone of CP-2 resulted in the detection of two CP-2 mRNA transcripts, 1.7 kb and 2.2 kb (Fig. 2), in agreement with data reported by Erickson-Lawrence et al. (1991). The 1.7 kb transcript was significantly more abundant than the 2.2 kb mRNA in the testicular samples while the 2.2 kb transcript was more heavily expressed in total RNA prepared from kidney (positive control; Fig. 2a). Treatment of animals with 650 mg/kg MAA led to the selective depletion of pachytene spermatocytes at all stages of the spermatogenic cycle except at early to mid stage VII. At 3 days after MAA treatment, all stages of the spermatogenic cycle, with the exception of stages I and VIII–XI but including stages IV–VII when the mRNA for CP-2 is normally expressed, were depleted selectively of pachytene spermatocytes. Depletion of these germ cells caused an apparent increase in the amount of mRNA for CP-2 in total testicular RNA when compared with controls (see Fig. 2a). At 7 days after MAA treatment, pachytene spermatocytes at stages VIII–XIII and round spermatids at stages II–V were depleted from the seminiferous tubules whilst tubules at stages I–II and VI–VII of the cycle had a full complement of germ cells. Similar to results obtained for MAA + 3 days, at 7 days after MAA-treatment there was an apparent small increase in CP-2 mRNA expression relative to intact controls (Fig. 2). At 14 days after MAA treatment when round spermatids were depleted from tubules at stages I–VI/VII (including stages at which CP-2 is normally expressed; see Fig. 1) and step 9 to step 15 spermatids were missing from stages IX-I a

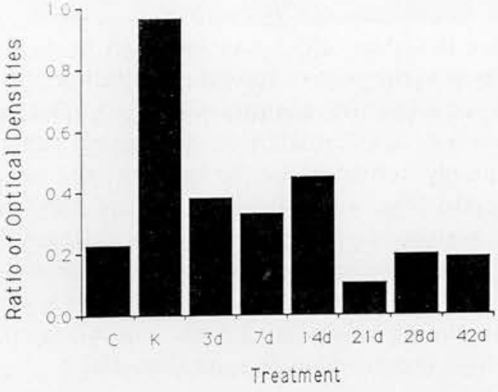

Fig. 3. Optical density of signals from Northern blot analysis (for panels A and B from Fig. 2) measured using image analysis. The ratios of CP-2 mRNA expression to 18S rRNA loading are plotted for each MAA time point to show the effect of treatment. C, control testis; K, kidney; 3, 7, 14, 21, 28 and 42 days after MAA treatment.

more marked increase in CP-2 mRNA expression on Northern blot was observed (Fig. 2). In contrast, at 21 days after MAA treatment, when elongate spermatids from stages I–VI/VII of the cycle (including stages IV–VII at which CP-2 is usually expressed heavily) and round/elongating spermatids at stages VII–XII/XIII were depleted selectively (Fig. 1) there was a major reduction in the signal for the 1.7 kb transcript for CP-2 (Fig. 2a). Levels of this transcript had returned almost to control levels at 28 days after MAA treatment when elongate spermatids were depleted only from stages I–IV/V of the cycle (Fig. 1) and results comparable to controls were obtained at 42 days after MAA treatment when all tubules had a full germ cell complement.

The comparative levels of CP-2 mRNA at each treatment time were calculated by densitometric measurements of the signal obtained by Northern blot. Any effect of unequal gel loading was overcome by measuring the level of 18S rRNA (Fig. 2b) and calculating the ratio of CP-2/18S RNA (Fig. 3).

Transition protein-2 (TP-2) is a DNA binding protein expressed specifically in the testis. The mRNA for TP-2 has been localised in step 7 to step 13 spermatids during their elongation phase (Saunders et al., 1992b). This probe has been used as a marker with which to confirm maturation depletion of germ cells caused by MAA treatment. When membranes were stripped and reprobed with a cDNA probe specific to TP-2 one abundant mRNA species of approximately 0.9 kb was visualised (Fig. 2c). The level of expression of this transcript remained constant in all MAA treated animals except at 14 and 21 days post treatment. At these time points TP-2 mRNA was significantly reduced or absent from the testis confirming the selective depletion/reduction of step 7–13 spermatids from the tubules at these times.

Cellular localisation of CP-2 mRNA

The mRNA for CP-2 was localised by in situ hybridisation to the base of seminiferous tubules by means of a non-radioactive riboprobe (Fig. 4). The level of expression of CP-2 mRNA in the Sertoli cells varied considerably according to the stage of the spermatogenic cycle (Fig. 4A). Highest levels of CP-2 mRNA expression were evident in Sertoli cells at stages IV–VII of the spermatogenic cycle, whilst there was no expression detectable at other stages. The mRNA was confined to the cytoplasm of Sertoli cells when observed under high power magnification (Fig. 4B).

Regulation of CP-2 mRNA

To extend the data gained from Northern analysis on the possible influence of different germ cell types on expression of CP-2 mRNA, testicular sections from animals at 3, 7, 14, 21, 28 and 42 days after MAA treatment were subjected to in situ hybridisation using digoxigenin- or ^{35}S-labelled riboprobes for CP-2. In testes from control animals, CP-2 mRNA expression was observed in 37% of tubules (obtained by counting 100 tubules at random from sections from each of two animals). At 3 days after MAA treatment, depletion of pachytene spermatocytes at those stages which normally express CP-2 mRNA (i.e. stages IV–VII) did not appear to have any significant effect on the level of expression of the transcript (Fig. 5) or on the frequency (33%) of tubules expressing CP-2 mRNA. At MAA + 7 days, pachytene spermatocytes at stages VIII–XIII and round spermatids at stages II–V were depleted from the seminiferous tubules. At this time point, tubules in which CP-2 mRNA is normally expressed, stages IV–V (missing round spermatids) and stages VI–VII (full germ cell complement), were still expressing the mRNA for CP-2 (37% of tubules were expressing; data not

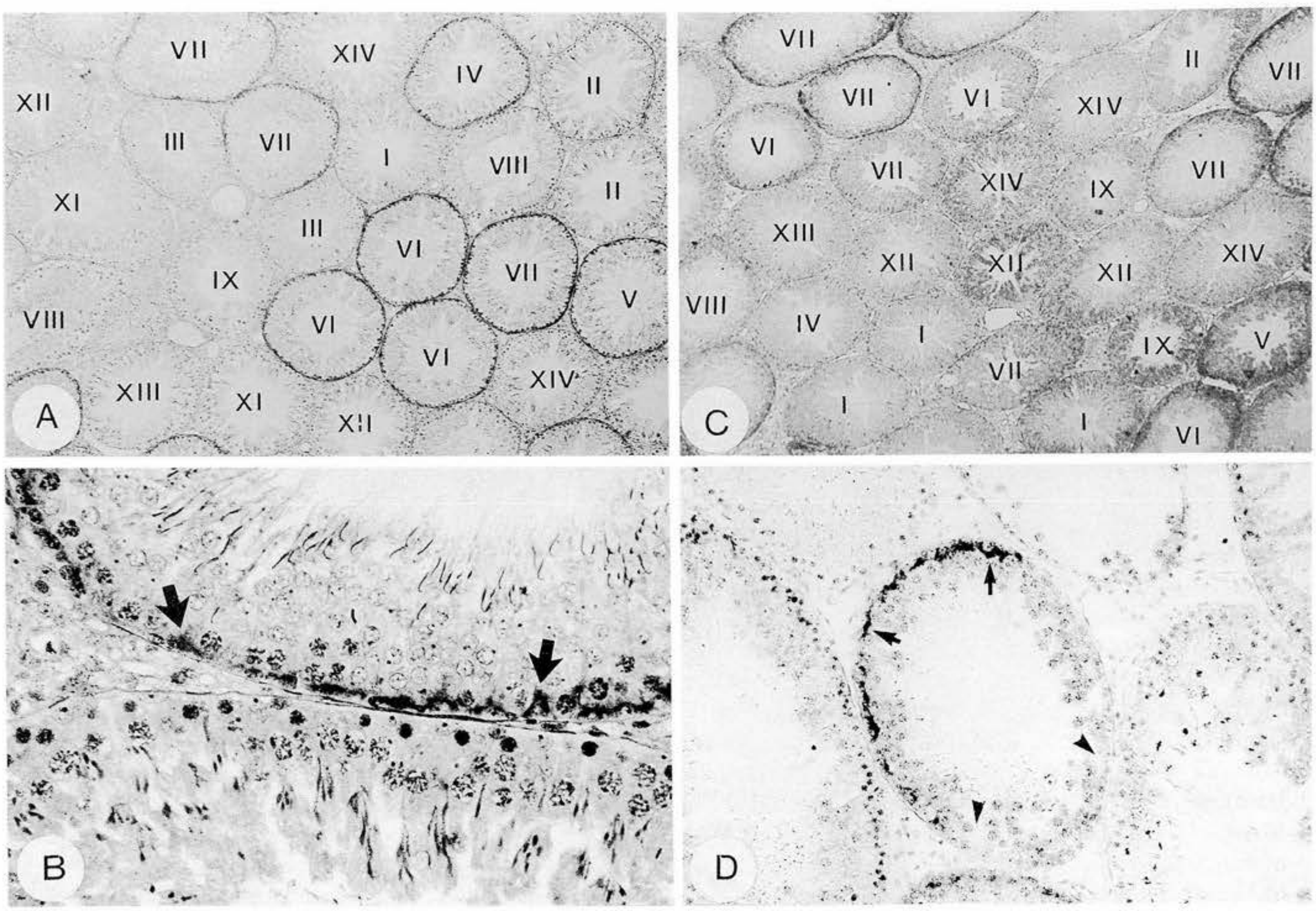

Fig. 4. Photomicrographs of cross-sections of perfusion fixed rat testis. Tissue was embedded in paraffin wax and sections cut at 5 μm. The tissue was probed with Dig-labelled riboprobes antisense to CP-2 mRNA. Sections were counterstained in haematoxylin after processing. (A) Control rat testis showing stage specificity of CP-2 mRNA expression. Tubules expressing CP-2 mRNA are at stages IV–VII of the spermatogenic cycle. Magnification × 40. (B) Stage VII tubule from control testis expressing CP-2 mRNA and showing subcellular localisation of mRNA in the Sertoli cell (arrows). Magnification × 245. (C) Section from testis 21 days after MAA treatment showing decrease in frequency and intensity of CP-2 mRNA expression compared to control. Magnification × 40. (D) Stage VII tubule from the testis of a rat 21 days after MAA-treatment and in which only part of the epithelium was depleted of elongate (step 19) spermatids. Sertoli cells remaining associated with elongate spermatids show CP-2 mRNA expression (arrows) while those with no associated elongate spermatids show no expression (arrowheads). Magnification × 100.

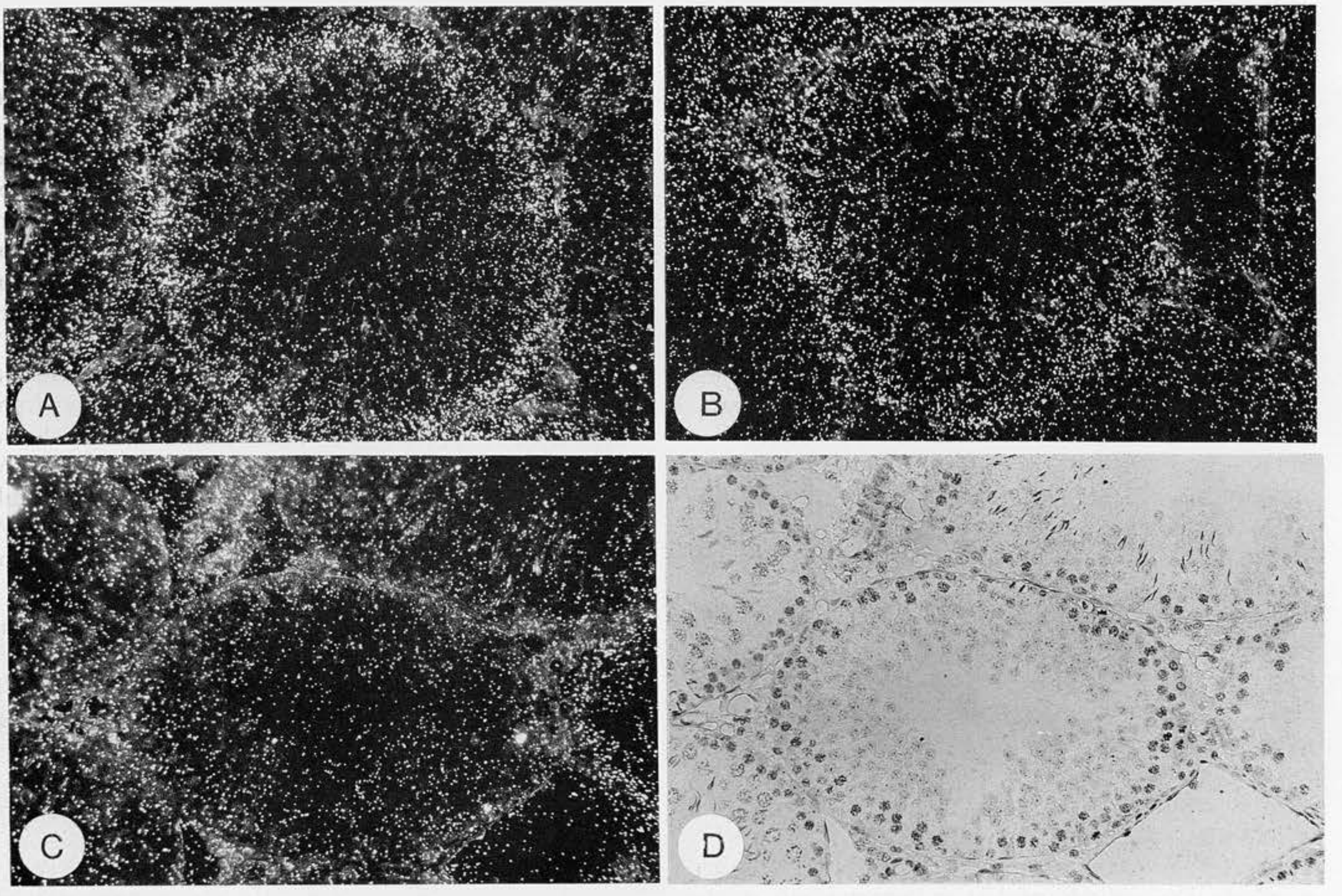

Fig. 5. Darkfield photomicrographs of sections probed with a ^{35}S-labelled riboprobe for CP-2. Tissue processing was as in Fig. 3. (A) Control section showing expression in a tubule at stage IV with a full complement of germ cells. (B) Section from an animal at 3 days after MAA treatment when pachytene spermatocytes were depleted from the illustrated stage IV tubule. (C) Section from an animal at 21 days after treatment with MAA showing loss of expression in a stage IV tubule from which elongate spermatids had been depleted. (D) Brightfield photomicrograph of section in panel C showing germ cell types present in the tubule. Magnification × 100.

shown). The depletion of round spermatids from tubules at stages when CP-2 is expressed (MAA + 14 days), except for stage VII which had a full complement of germ cells, did not lead to any change in the frequency of tubules showing a positive hybridisation signal (36%; data not shown). In contrast, at 21 days after MAA treatment the amount of mRNA visualised by in situ hybridisation with the CP-2 riboprobe was significantly different from those of controls (see Figs. 4, 5). The frequency of seminiferous tubules showing a positive hybridisation signal was reduced (22% in MAA + 21 day compared to 37% in controls). Tubules at stages IV to early-midstage VII had been depleted of their elongate spermatids (step 17 to step 19 spermatids) and no longer expressed CP-2 mRNA. However, certain tubules at this time point did still express CP-2 mRNA. Morphological examination of these tubules revealed that both their round and their elongate spermatids were depleted; these tubules were thought to be at late stage VII of the spermatogenic cycle (see Ratnasooriya and Sharpe, 1989). At 28 days after MAA treatment, step 15 to step 17 spermatids were depleted from tubules at stages I–IV/V. CP-2 mRNA expression was not detectable in stage IV–early stage V while normal expression remained in late stage V–stage VII. There was no significant difference in the frequency of tubules expressing mRNA for CP-2 at this time point when compared to controls (30% in MAA + 28 days; 37% in controls). At 42 days after treatment, tubules at stages when CP-2 mRNA is normally expressed had a full complement of germ cells and there was no difference in the frequency (33%) or the level of expression at this time point when compared to controls.

Discussion

The present study has used in situ hybridisation and a digoxigenin labelled riboprobe to localise CP-2 mRNA to Sertoli cells within the rat seminiferous epithelium and to assess the stage specificity and germ cell-modulation of expression of this mRNA. To our knowledge these are the first such studies involving in situ localisation of the CP-2 mRNA. Expression of the CP-2 mRNA was shown to be stage specific; the mRNA was expressed only in tubules from stage IV to stage VII of the spermatogenic cycle. A similar pattern of expression has been shown by Northern analysis of isolated tubules at different stages of the spermatogenic cycle (Erickson-Lawrence et al., 1991). These authors observed maximal expression of CP-2 mRNA at stages VI and VII, decreased expression at stage VIII and no detectable expression at stages XII and II. The pattern of expression of CP-2 mRNA correlates well with the pattern of expression of its protein as reported previously by Zabludoff et al. (1990). These workers localised CP-2 protein by immunohistochemistry and found that it was only detectable in Sertoli cells of seminiferous tubules at stages V–VIII.

The major finding of the present study was the modulation of CP-2 mRNA levels by elongate spermatids. CP-2 mRNA expression was shown both by Northern blot and in situ analysis to be reduced in testes recovered from animals at 21 days after treatment with MAA compared with controls. At this time point, seminiferous tubules at most stages of the spermatogenic cycle, with the exception of stages XIII–XIV and late stage VII to stage VIII, were depleted of elongating (steps 8–12) or elongated (steps 15–18) spermatids. At 21 days after MAA-treatment, tubules at stages IV–early stage VII at which there would normally be expression of CP-2 mRNA did not show a positive signal when examined by in situ hybridisation. These tubules were depleted of elongate spermatids but retained their spermatogonia, pachytene spermatocytes and round spermatids. The results are therefore consistent with the full expression of CP-2 mRNA being dependent upon the presence of elongate spermatids. In contrast to these results, at 28 days after MAA treatment no significant change in the level of mRNA for CP-2 was observed, although elongate spermatids were depleted from tubules at stages I–IV and early stage V of the spermatogenic cycle. The frequency of occurrence of tubules at stages IV (4.8%) and V (6.8%) in the testis is very low compared to that of stage VII (20.9%; Hess et al., 1990). Therefore the effect of depletion at this point is not apparent on Northern analysis of total testicular RNA as at 21 days after MAA treatment when stages IV to early- to mid-stage VII were all depleted of elongate spermatids. In situ hybridisation of tissue recovered 28 days after MAA treatment did confirm the loss of CP-2 mRNA expression in tubules at stages IV and V which were lacking elongate spermatids.

This study has also shown that at 21 days after MAA treatment the expression of mRNA for CP-2 was maintained in some tubules which were depleted of elongate spermatids. Using brightfield microscopy, it was determined that these tubules were depleted not only of elongate spermatids but also of round spermatids. These tubules were determined to be at late stage VII based on a number of criteria such as the presence of preleptotene spermatocytes and the pattern of germ cell depletion calculated to occur at this time (Bartlett et al., 1988; Ratnasooriya and Sharpe, 1989). Therefore, we speculate that round spermatids may also be involved in modulating Sertoli cell expression of CP-2 mRNA at certain stages of the spermatogenic cycle. In support of this idea Northern blot analysis showed an apparent increase in the amount of CP-2 mRNA in testes recovered from animals 7 and 14 days after MAA treatment, times at which round spermatids were depleted from tubules at most of the stages (IV–VII) at which CP-2 mRNA is normally expressed. The frequency of tubules expressing CP-2 mRNA at these time points, as observed by in situ hybridisation, did not differ from that of controls. It is possible that the level of CP-2 mRNA expressed at stages IV–VII of the spermatogenic cycle in sections from animals at 7 and 14 days after MAA treatment is increased compared to controls. Unfortunately, quantification using the available autoradiography software packages for image analysis has proved impossible due to the discrete localisation of the signal around the base of the seminiferous tubules. However, it has also been shown by two-dimensional gel electrophoresis that depletion of round spermatids due to MAA treatment causes an apparent increase in secretion of CP-2 protein by isolated seminiferous tubules at stages VI–VIII compared to that secreted by tubules at the same stages isolated from control animals (Sharpe et al., 1992). These observations lead us to speculate that CP-2 production may also be regulated (negatively) at the level of transcription in the adult seminiferous tubule.

It has been shown previously that CP-2 secretion by seminiferous tubules increases in the developing testes as the number of round spermatids increases (Zabludoff et al., 1990). This may indicate that in contrast to the findings of the present study, round spermatids stimulate rather than inhibit CP-2 production during pubertal growth of the rat testis. Evidence has been presented in support of the idea that the response of the Sertoli cell to germ cells is dependent on the stage of maturation of the Sertoli cells. For example, it has been shown that round spermatids cause an increase in the levels of α-inhibin mRNA when cultured with Sertoli cells isolated from 20-day-old rats (Pineau et al., 1990). In contrast, secretion of α-inhibin in vivo by Sertoli cells in adult rats decreased only in the absence of elongate spermatids while it was unaffected by depletion of round spermatids (Allenby et al., 1991). It has been hypothesised that modulation of Sertoli cell function during development of the testis is transferred sequentially to the most advanced generation of germ cells present within the seminiferous epithelium (Jégou, 1991). A similar situation may apply to the regulation of CP-2 production in the developing rat testis compared to that found in the mature animal.

Several studies have implicated spermatids in the modulation of various aspects of Sertoli cell function. Addition of round spermatids to immature Sertoli cells in culture has been shown to cause an increase in the secretion of androgen binding protein (ABP; Le Magueresse et al., 1986) as well as α-inhibin (Pineau et al., 1990). Maturation depletion of germ cells, initiated by gamma-irradiation of the testis, showed a strong correlation between ABP production and the presence of elongate spermatids (Pineau et al., 1989) while depletion of elongate spermatids from the tubules following MAA treatment led to a decrease in secretion of immunoreactive α-inhibin in vivo and in vitro (Allenby et al., 1991) as previously stated. Elongate spermatids have also been reported to play a role in regulating the production of seminiferous tubule fluid by the Sertoli cell (Jégou et al., 1984), the volume of the lumen of the seminiferous tubule (Sharpe, 1989) and overall protein secretion (McKinnell and Sharpe, 1992). The modulation of CP-2 mRNA levels by elongate spermatids, and possibly also by early spermatids as shown here, is therefore yet another example of germ cell modulation of Sertoli cell function.

Northern blot analysis also indicated an apparent increase in CP-2 mRNA levels at 3 days after MAA treatment. At this time point, tubules at all stages of the spermatogenic cycle, with the exception of stages I and VIII–XI, were depleted of most pachytene spermatocytes. It is well established that pachytene spermatocytes are very active transcriptionally, and contain larger amounts of mRNA than do other testicular cell types (Monesi et al., 1978). It is therefore possible that depletion of the majority of pachytene spermatocytes at this time point leads to a much higher percentage of Sertoli cell RNA being loaded in lanes from rats at MAA + 3 days compared to that in the control lanes, as equal amounts of total RNA were loaded onto each lane of the denaturing agarose gel. This interpretation is supported by the absence of any obvious change at 3 days after MAA treatment in the mRNA level for CP-2 when assessed by in situ hybridisation. Furthermore, the absence of pachytene spermatocytes has no effect on the secretion of CP-2 protein by isolated seminiferous tubules (C. McKinnell and R.M. Sharpe, unpublished data). However, we cannot rule out completely the possibility that pachytene spermatocytes also exert a modulatory effect on CP-2 gene expression.

Substrates for the catalytic activity of cathepsin L (CP-2) which include several extracellular matrix proteins have been identified in various tissues (Kirschke et al., 1982; Gal and Gottesman, 1986; Johnson et al., 1986; Mason et al., 1986). However, the exact function of this protease within the testis remains unknown. It has been proposed (Erickson-Lawrence et al., 1991) that CP-2 is involved in the breakdown of interactions between spermatids and Sertoli cells to allow detachment/movement of the spermatids from the deep recesses in the Sertoli cell at stages IV–V to the luminal edge at stages VI–VIII. The data presented within this paper are consistent with control of this movement being initiated by the elongate spermatids themselves, although the mechanism by which this modulation occurs is as yet unclear.

In conclusion, the present study has shown that expression of CP-2/cathepsin L mRNA by Sertoli cells is modulated by spermatids, in particular by elongate spermatids at stages IV–VII of the spermatogenic cycle. The mechanism by which the spermatids control expression has not been identified. It is proposed that the spermatids may regulate their own movement within and release from the seminiferous epithelium by modulating activity of the CP-2 gene and hence the production of CP-2 by Sertoli cells.

References

Allenby, G., Foster, P.M.D. and Sharpe, R.M. (1991) Endocrinology 128, 467–476.

Bartlett, J.M.S., Kerr, J.B. and Sharpe, R.M. (1988) J. Androl. 9, 31–40.

Chambers, A.F., Collela, R., Denhart, D.T. and Wilson, S.M. (1992) Mol. Carcinogenet. 5, 238–245.

Chang, Y.L., Gutell, R., Noller, H.F. and Wool, I.G. (1984) J. Biol. Chem. 259, 224–230.

Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.

Davis, L.G., Dibner, M.D. and Battey, J.F., eds. (1986) Basic Methods in Molecular Biology. Elsevier, New York.

Erickson-Lawrence, M., Zabludoff, S.D. and Wright, W.W. (1991) Mol. Endocrinol. 5, 1789–1798.

Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.

Gal, S. and Gottesman, M.M. (1986) J. Biol. Chem. 261, 1760–1765.

Hess, R.A., Schaeffer, D.J., Eroschenko, V.P. and Keen, J.E. (1990) Biol. Reprod. 43, 517–524.

Jégou, B. (1991) Ann. N.Y. Acad. Sci. 637, 340–353.

Jégou, B., Laws, A.O. and De Kretser, D.M. (1984) Int. J. Androl. 7, 244–257.

Johnson, D.A., Barrett, A.J. and Mason, R.W. (1986) J. Biol. Chem. 261, 14748–14751.

Kirschke, H., Kembhavi, A.A., Bohley, P. and Barrett, A.J. (1982) Biochem J. 201, 367–372.

Leblond, C.P. and Clermont, Y. (1952) Ann. N.Y. Acad. Sci. 55, 548–573.

Le Magueresse, B., Le Gac, F., Loir, M. and Jégou, B. (1986) J. Reprod. Fertil. 77, 489–498.

Le Magueresse, B. and Jégou, B. (1988a) Mol. Cell. Endocrinol. 58, 65–72.

Le Magueresse, B. and Jégou, B. (1988b) Endocrinology 122, 1672–1680.

Mason, R.W., Barrett, A.J. and Chapman, H.A. (1986) Biochem. J. 233, 925–927.

McKinnell, C. and Sharpe, R.M. (1992) Mol. Cell. Endocrinol. 83, 219–231.

Millar, M.R., Sharpe, R.M., Maguire, S.M. and Saunders, P.T.K. (1993) Cell Tissue Res., in press.

Monesi, V., Geremia, R., D'Agostino, A. and Boitani, C. (1978) Curr. Topics Dev. Biol. 12, 11–36.

Onoda, M. and Djakiew, D. (1990) Mol. Cell. Endocrinol. 73, 35–44.

Parvinen, M. (1992) in The Sertoli Cell (Russell, L.D. and Griswold, M.D., eds.), Cache River Press, in press.

Pineau, C., Sharpe, R.M., Saunders, P.T.K., Gerard, N. and Jégou, B. (1990) Mol. Cell. Endocrinol. 72, 13–22.

Pineau, C., Velez de la Calle, J.F., Pinon-Lataillade, G. and Jégou, B. (1989) Endocrinology 124, 2720–2728.

Ratnasooriya, W.D. and Sharpe, R.M. (1989) Int. J. Androl. 12, 44–57.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Science 239, 487–491.

Saunders, P.T.K., Padayachi, T., Tincello, D., Shalet, S.M. and Wu, F.C.W. (1992a) Clin. Endocrinol. 37, 214–220.

Saunders, P.T.K., Millar, M.R., Maguire, S.M. and Sharpe, R.M. (1992b) Mol. Reprod. Devel. 33, 385–391.

Sharpe, R.M. (1989) J. Androl. 10, 304–310.

Sharpe, R.M. (1992) in The Sertoli Cell (Russell, L.D. and Griswold, M.D., eds.), Cache River Press, in press.

Sharpe, R.M., McKinnell, Millar, M., Maguire, S. and Saunders, P.T.K. (1992) in Understanding Male Infertility: Basic and Clinical Approaches (Whitcomb, R. and Zirkin, B., eds.), Raven Press, New York, in press.

Wright, W.W., Parvinen, M., Musto, N.A., Gunsalus, G.L., Phillips, D.M., Mather, J.P. and Bardin, C.W. (1983) Biol. Reprod. 29, 257–270.

Zabludoff, S.D., Erickson-Lawrence, M. and Wright, W.W. (1990) Biol. Reprod. 43, 15–24.